STUDIES ON AN ENDONUCLEASE FROM GERMINATING PEA (PISUM SATIVUM) SEEDS.

By

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SUMMARY AND CONCLUSION

1. A nuclease activity was identified in the crude extracts of germinating pea seeds that degraded denatured, alkylated and depurinated DNA at a significantly higher rate than native DNA. The activity was found to be present in dry and imbibed seeds. There was no significant change in its specific activity over a germination period of 120 hours.

2. The enzyme was purified about 150-fold on the basis of its ability to degrade single stranded DNA, using ammonium sulfate fractionation, DEAE-cellulose and Phosphocellulose chromatography. The final Phosphocellulose fraction (Fraction IV) was not homogeneous as three discrete protein bands were observed on disc gel electrophoresis.

3. From exclusion chromatography on sephadex G-200 the molecular weight and Stoke's radius of pea seed nuclease was calculated to be 42,000 and 5.19×10^{-7} cm.

4. The partially purified enzyme degraded single stranded DNA at a 15 to 20 fold higher rate than native DNA. The hydrolysis of single stranded and depurinated DNA by partially purified enzyme was found to be at comparative levels. That the activity of the purified preparation on double and single stranded DNA is due to a single enzyme

is suggested by several criteria.

5. The Km value for the enzyme as determined in terms of acid soluble DNA nucleotides formed by hydrolysis of single stranded DNA was of the order 3.33×10^{-6}M.

6. The enzyme shows activity over a broad range of pH but was most active between pH 6.5 to 8.0. At pH 7.5 the enzyme was maximally active in tris-HCl buffer, while in other buffers of same molarity and pH the enzyme shows decreased activity.

7. A broad range of temperature tolerance was shown by the enzyme. The maximum hydrolysis of denatured DNA was observed at 45° while with native DNA the temperature optima was 60°. The enzyme is fairly stable and retains unchanged activity when stored frozen in the presence of 10% glycerol in 0.02M tris-HCl buffer, pH 7.5. Slow decrease in enzyme activity was observed as a function of time when enzyme was incubated at 37° and 45°. However at 50°, half of the activity was lost within two hours.

8. The small extent of hydrolysis of native DNA is suggested to be due to the degradation of partially denatured areas in the native molecule and not due to a contamination by single stranded molecules in the native DNA preparation. This was concluded on the basis of experiments in which

native DNA when treated with nuclease at elevated temperatures, showed increased hydrolysis both in extent and rate. This indicated that the enzymatic activity was directed towards low melting or A, T-rich regions in native DNA. Higher ionic strength suppresses the structural breathing in native DNA. The enzymatic activity of the nuclease on native DNA was found to be reduced by approximately 90% in the presence of 0.2M NaCl. This property further supported the idea that the degradation of native DNA by the enzyme was mainly directed towards partially denatured areas.

9. The nuclease does not show an absolute requirement for added divalent cations. However the addition of Mg^{++} and Ca^{++} results in 40% and 60% stimulation respectively. Among the chelating agents 8-hydroxyquinoline was inhibitory whereas EDTA had no effect upon the enzymatic activity. The observed inhibition of the pea seed nuclease in presence of 8-hydroxyquinoline but not with EDTA as well as the lack of an absolute requirement for the added divalent cation suggest that free metal is not required for enzyme function and the inhibition by 8-hydroxyquinoline is probably through direct interaction with the enzyme possibily with a bound metal. Combined addition of Ca^{++} and Mg^{++} to the reaction mixture did not show synergistic effect. EDTA causes the

reversal of stimulation by Ca^{++} and Mg^{++}. The enzyme does not seem to require an -SH group for its activity. Addition of yeast RNA and several mononucleotides did not show inhibition of enzyme activity.

10. The study of products of enzymatic hydrolysis of denatured DNA on sephadex G-100 show that oligonucleotides are produced during the course of reaction and are progressively reduced to smaller fragments untill virtually the entire population of single stranded DNA is rendered acid soluble. The chromatographic profile obtained with the digests of enzymatic hydrolysis was consistant with an endonucleolytic mode of action.

11. The preferential degradation of alkylated DNA by both crude as well as purified enzyme was further investigated by studying the effect of alkylating agents on the secondary structure of DNA. Native DNA on its treatment with dimethyl sulfate undergoes strand separation as determined by hydroxyapatite chromatography. The extent of denaturation depends upon the DNA nucleotide/DMS molar ratio. However, the presence of a higher salt concentration has a preventive effect on alkylation induced denaturation. The hydrolysis of alkylated DNA by the enzyme is due to the denaturing effect of the alkylating agent.

12. Cross linked DNA is resistant to the action of pea seed nuclease. Since cross linking may presumably suppress the single stranded character of native DNA this fact further strengthens the indication that nuclease recognizes the partially denatured areas in the native molecule.

The importance of single strand specific deoxyribonucleases has been recognized in many laboratories. The higher selectivity of pea seed nuclease towards single stranded DNA (whether modified or otherwise) and denatured or low melting regions in native DNA, stability at a wide range of temperatures and ionic strength, a neutral pH optima and ease of preparation of this nuclease endow this enzyme with optimal qualities required for a nuclease to be used as an analytical tool. With respect to its physiological role the pea seed nuclease may be implicated in several processes of biological importance such as DNA repair, replication and recombination. As a biochemical tool it complements the other single strand DNA specific nucleases such as the mung bean nuclease and S_1 nuclease of Aspergillus oryzae that have already been reported to be of wide utility.

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I certify that the work presented in this thesis has been carried out by Mr. Altaf Ahmad Wani and is suitable for the award of Ph.D. degree in Bio-Chemistry of the Aligarh Muslim University, Aligarh.

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DEDICATED TO
MY PARENTS AS A SYMBOL OF GRATITUDE

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CHAPTER - I

I N T R O D U C T I O N

INTRODUCTION

Study of nucleases has been of considerable interest in the past, particularly their purification, specificity and mode of action to gain an insight into the ways in which cell maintains its own continuity by preserving the integrity of genetic material in the presence of a multiplicity of degradative agents. Experiments carried out with ingenuity have revealed the ubiquitous existence of a number of nucleases that perform the degradation of DNA of one or other kind; and have been categorized according to the nature of their attack. Endonucleases that hydrolyse the phosphodiester bond of DNA liberating oligonucleotides have been purified from bacteria, molds, plants and animals. Exonucleases liberate mononucleotides in rapid succession, and they vary further in the nature of substrate they attack and kind of product they form. Some of them are implicated in specialized processes performing interestingly important roles of genetic and physiological significance.

GENERAL SURVEY OF NUCLEASES

There is considerable evidence that deoxyribonucleases perform an accessory function in DNA replication[1]. Repeatedly it has been observed that nucleases can profoundly affect the structure of the template, hence the rate of replication and nature of the product synthesized. Support for the notion that deoxyribonucleases may be involved in DNA synthesis has essentially come from three sources; (a) from *in vivo* studies with a wide variety of organisms which have shown that cellular deoxyribonuclease levels are generally, though not invariably highest during that interval in the growth cycle when DNA synthesis is proceeding at maximal rates; (b) from the appearance, specifically in response to viral infection, of a large number and variety of deoxyribonucleases. In certain instances these enzymes may well function in the degradation of host DNA to provide the large pool of precursors demanded by the rapid virus induced synthesis of DNA; on the other hand virus specific deoxyribonucleases, often in higher concentration, have been identified in situations where little if any breakdown of host DNA occurs; and (c) from studies on DNA replication by purified DNA polymerases in which it has been observed repeatedly that nucleases can profoundly effect the structure of the template, hence the rate of

replication and the nature of the product synthesized. Further, two of the DNA polymerases[2,3] that have been isolated as physically homogenous proteins possess exonuclease activity which cannot be separated physically from the polymerase activity. It is proposed that the coexisting exonuclease activity empowers the DNA polymerase to replicate two strands of DNA duplex, by inflicting nicks in the twisted DNA and at the growing replicating fork.

Current models of genetic recombination[4] suggest several roles for deoxyribonucleases. Endonucleases are necessary to introduce the initial single strand or double strand breaks in the parent duplex. Although endonucleases are known which introduce both types of interruptions in the DNA molecules, there is no direct evidence available which implicates them in recombination. Exonucleases may be required for the exposure of additional single strand regions of sufficient length for recombination. Exonuclease II or III from E. coli[5,6] or phage exonuclease[7] are examples of enzymes which could accomplish this. The only deoxyribonuclease which has been positively identified in genetic recombination is the exonuclease of phage λ .

In addition to their involvement in DNA replication and recombination, the deoxyribonucleases may also serve the cell in what may be termed a 'protective' capacity. There are atleast

two cases in which it may occur.

(a) restriction of host modified DNAs as a means of protecting the cell against the intrusions of foreign genetic material; and

(b) excision of lesions introduced into DNA as a result of exposure to U.V. and other kinds of irradiation or to alkylating and other chemical agents, thus permitting the repair of the injured nucleic acids.

There is evidence that the modification properties of a DNA molecule are determined by its pattern of secondary methylation. The simplest hypothesis for biochemical basis of restriction is that each restriction character directs the formation of a nuclease specific for DNA lacking the corresponding modification. Such an endonuclease is present in strain K of E. coli[3] and is specific against DNA from strains lacking modification allele mk. This enzyme is called endonuclease R.K.

The system of host controlled modification and restriction provides unique defense mechanism for bacterial cell; DNA foreign to the strain is specifically identified and inactivated as a result of degradation by restriction endonucleases. This system is particularly effective towards a virus which has recently been introduced from a foreign environment, a virus to which the bacterial population has not yet built up an immunity.

Restriction nucleases have been studied in other strains of bacteria. In addition restriction enzymes of episomes such as drug resistant transfer factor RI and RII etc. have proved useful in the technique of Genetic engineering[9,10]. Although, this or an analogous restriction process has not as yet been identified in cells of higher organisms the existence of such a mechanism remains an intriguing possibility. The restriction activity provides the first endodeoxyribonuclease with an activity which is known to reflect its *in vivo* activity both in extent and specificity.

Genetic material is susceptible to a number of different kinds of damage. The integrity of high molecular weight of cellular DNA is maintained by enzyme systems contained in various organisms, which are thought to act upon the DNA containing single strand breaks, damaged bases or other structural defects. The mechanism that ensues may be different depending upon the kind of damage, but the ultimate aim is to do away with the offending product and reconstruct DNA in twin helical form.

Friedberg and Goldthwait[11,12] and Friedberg *et al.*[13] reported a nuclease known as endonuclease II from *E. coli* which specifically hydrolyses the phosphodiester bonds in alkylated DNA. The enzyme makes both double and single stranded breaks in DNA alkylated with monofunctional alkylating agent methyl methane sulfonate. Hadi and Goldthwait[14] purified the enzyme

and found it to be active upon partially depurinated DNA. Endonuclease II is not inhibited by tRNA and has no absolute requirement for metal ions. Verly et al.[15] and Rani and Nadi[16] have recently obtained evidence that the nuclease specific for apurinic sites in the DNA is present in rat liver and in plant extracts in addition to E. coli. According to Verly[17] and Verly and Paquette[18] rat liver contains an endonuclease which breaks DNA strands near apurinic sites, and the purified enzyme is strictly specific for apurinic sites and has no action on normal DNA. Lindahl and coworkers[19,20] have purified an endonuclease from calf thymus that specifically acts upon apurinic sites in double stranded DNA. They suggest that the enzyme catalyses the initial incision step, in the excision repair mechanism for the repair of apurinic sites. Kirtikar et al.[21] have reported that endonuclease II comprises of two nucleases; one specific for depurinated DNA and other for methyl methane sulfonate alkylated DNA. The latter has been shown to be active on γ-irradiated DNA[22]. There is as yet no direct evidence for the existence of additional DNA endonucleases specifically attacking other types of lesions than pyrimidine dimers or apurinic sites.

Much work has been done on the nucleases of E. coli, an organism in which a possible enzymatic pathway of DNA synthesis has been described[23] and whose genetic structure has been under

intensive physiological and chemical investigation. Secondly these enzymes are capable to attack DNA in a variety of ways and thus play an important role in the nucleic acid economy of the bacterium. Thirdly deoxyribonucleases of defined specificity could serve as useful reagents in determining the structure and deoxyribonucleotide sequence of the DNA molecule.

There are a number of enzymes present in cell free extracts of E. coli which can degrade DNA and smaller polynucleotides derived from it[24-26]. One of these enzymes E. coli exonuclease I[27] hydrolyses E. coli and Calf Thymus DNAs to their 5'-mononucleotides, once these polymers have undergone some degradation as a result of either heating or limited treatment with pancreatic deoxyribonuclease. Study of the capacity of the extracts of E. coli to depolymerize DNA under conditions which effectively mask exonuclease I and endonuclease I revealed the existence of yet another deoxyribonuclease activity[28]. This activity has been purified extensively in parallel with the DNA polymerase and is infact physically inseparable from it even in the most highly purified preparations[29]. This was attributed to exonuclease II which persues a purely exonucleolytic course of hydrolysis. Like exonuclease I it attacks at the 3'-hydroxyl terminus of polydeoxyribonucleotide chain, successively liberating deoxyribonucleoside-5'-monophosphates. Unlike exonuclease I which is unable to catalyze the hydrolysis of dinucleotides

exonuclease II quantitatively degrades polynucleotides to their component mononucleotides. In further contrast to exonuclease I, exonuclease II attacks native DNA and at a several fold greater rate than denatured DNA. In addition to exonuclease I and II, a third exonuclease III activity is present in E. coli and is shown to be closely associated or identical with a DNA specific phosphatase[30]. This activity is described by Richardson, Lehman and Kornberg[6,31]. It attacks 3'-hydroxyl or 3'-phosphate terminus of double stranded DNA degrading it to deoxyribonucleoside-3'-monophosphates. Though highly specific for bihelical DNA, it can also hydrolyse 3'-monoester groups that terminate long deoxyribonucleotides. Hydrolysis by this enzyme is apparently initiated at the two opposite 3'-hydroxyl ends of the DNA molecule. Half of each strand is degraded to mononucleotides and the remaining halves are single strands that are resistant to further hydrolytic action.

E. coli contains a deoxyribonuclease activity which attacks oligonucleotides 20 times more rapidly than either native or heat denatured DNA[32,33]. In the process of purifying E. coli deoxyribonucleotidase it was observed that there are two chromatographically distinct fractions which exhibit this substrate preference. Since the degradation of oligonucleotides by these enzyme preparations appears to proceed in an exonucleolytic fashion it was termed E. coli exonuclease-IV, more specifically the oligonucleo-

tidase activity found in the early fractions of chromatographic effluents has been designated exonuclease-IV A whereas the oligonucleotidase activity eluted at a higher ionic strength has been designated exonuclease-IV B previously described as exonuclease V. Both fractions of oligonucleotidases are similar to the other exonucleases of E. coli in that they produce 5'-mononucleotide products and are maximally active at slightly alkaline pH.

Contrary to the action of the nucleases which attack 3'-terminus exonuclease VI of E. coli attacks 5'-hydroxyl or 5'-phosphate terminus of double stranded DNA producing deoxyribonucleoside-3'-monophosphates[35]. It is optimally active at neutral pH and causes preferential degradation of dAT homopolymer.

Another class of E. coli enzymes that persue an endonucleolytic mode of degradation include endonuclease I and endonuclease II. Endonuclease I attacks both native and denatured DNA yielding products of which all are larger than pentanucleotides.[36,37] This enzyme produces scissions at many points along the DNA chain. Studier[38] and Bernardi and Cordonnier[39] have shown that attack of bihelical DNA occurs in such a way as to produce double stranded breaks in the molecule.

Two deoxyribonucleases have been isolated from pneumococcus[40] an exonuclease and an endonuclease. The exonuclease is active on both native and denatured DNA producing 5'-phosphoryl terminated oligonucleotides.

Trilling and Aposhian[41] have described an activity in Bacillus subtilis 80 19 infected with phage SP_3, which degrades heat denatured DNA to acid soluble components. Of the acid-soluble products formed during hydrolysis of denatured DNA by purified enzyme, 90% are dinucleotides and 10% are trinucleotides[42]. Since large acid soluble fragments are not formed it appears that the enzyme is an exonuclease initiating hydrolysis at the 3'-end and releasing di or trinucleotides in a sequential manner.

According to Linn and Lehman[43,44] Neurospora crassa contains an endonuclease that preferentially degrades denatured DNA and RNA. The hydrolysis of native DNA by the enzyme has been attributed to a contaminating nuclease which can be removed by a variety of treatments. S_1 nuclease of Aspergillus oryzae[45] and the nuclease from Ustilago maydis[46] have been shown to be specific for single stranded DNA.

Report of an apparently labile nuclease by Masui, Hara and Hiramatsu[47] from soybean sprouts led to the discovery of an important nuclease from mung bean sprouts[48], which degrades heat denatured DNA and has no activity upon unheated DNA.

Plant nuclease I from tobacco cell cultures[49] has been reported to be an endonuclease with associated 3'-nucleotidase activity and is competitively inhibited by ATP. In Avena leaf tissue an enzyme is described[50] which has similar specificity towards both RNA and DNA and accumulates during leaf senescence.

The enzyme is an endonuclease which hydrolyses both single and double stranded structures including double stranded DNA, however, the rate of hydrolysis was higher with single stranded DNA. Germinating wheat seedlings contain an enzyme which in highly purified form hydrolyses DNA, rRNA and the 3'-phosphodiester linkage of 3'-AMP at similar rates[31]. These three activities remained associated throughout a variety of purification procedures and evidence is provided that they probably reside in the single protein.

Enzyme similar in action to that present in Neurospora crassa, Aspergillus oryzae, and Ustilago maydis has been found in mouse ascites cells[52] and in Concanavalin-A proliferating lymphocytes[53,54]. The enzyme is highly specific for denatured DNA and is the first of its kind of endonuclease reported from mammalian sources. Besides DNase I and II reported from mammalian sources[55-57] rabbit bone marrow contains DNase III[58] that hydrolyses DNA in an exonucleolytic fashion from 3'-end of DNA, releasing 5'-mononucleotides as main degradative products. It does not attack RNA, has an alkaline pH optima and requires a divalent metal ion as a cofactor. The properties of DNase III from normal rabbit tissue are apparently similar to those of DNA exonuclease activity previously observed in tumor cell extracts from mouse mammary tissue[59,60], Novikoff ascites hepatoma cells of rats[61] and human Hela cells[62]. DNase III is often the most prevalent

DNase activity and most predominant DNA exonuclease observed in mammalian cell extracts at alkaline pH values, since DNase I is inhibited by a widely distributed specific protein inhibitor[63], DNase II and lysosomal exonuclease[64] have acid pH optima. Lindahl and coworkers describe the presence of DNase IV[65] that degrades native DNA exonucleolytically yielding 5'-mononucleotides, is usually present in smaller amounts in nuclei and cytoplasm. DNase III and IV as exonucleases are likely the enzymes involved in the repair of DNA in the mammalian tissues as described in bacteria[66]. Ca^{++} - Mg^{++} dependant endonuclease has been reported in rat liver[67] which is designated DNase V. It has a neutral pH optima and prefers double stranded DNA. Recently a novel endonuclease has been shown to be present in human aneuploid cell cultures that degrades single stranded DNA[68]. This enzyme designated DNase VI has an alkaline pH optima and requires Mg^{++} for its activity and has properties similar to the enzymes described in Ustilago maydis[69] and Bacillus subtilis[70] for which an essential role in recombination seems likely.

NUCLEASES DEGRADING SINGLE STRANDED DNA

Exonuclease I of E. coli has only slight activity with intact double stranded DNA, but has a high degree of selectivity for denatured DNA or DNA from bacteriophage ϕx174[27] which are

degraded to 5'-mononucleotides. Hydrolysis proceeds exonucleolytically in a stepwise manner beginning at the 3'-hydroxyl end of the chain. The enzyme degrades pancreatic limit digest of calf thymus DNA and is capable of degrading appropriately pretreated bacteriophage DNA bearing glycosylated hydroxymethyl cytosine to their constituent mononucleotides. In this respect it differs from venom diesterase[71] which is unable to catalyse the cleavage of most of the linkages in which glycosylated hydroxymethyl cytosine is involved[72-74]. The purified enzyme is most active at alkaline pH (9.3-9.8) and requires Mg^{++} for its optimal activity.

Exonuclease II of *E. coli*[5] carries out an exonucleolytic attack of DNA (native and denatured), deoxyadenylate - deoxythymidilate copolymer, and deoxyguanidylate-deoxycytidylate homopolymers, as well as on small oligonucleotides, hydrolysing these quantitatively to deoxyribonucleoside-5'-monophosphates. The maximum velocity constant observed for these substrates do not differ by more than 60 fold, but Michaelis constant for the small oligonucleotides are about 10^6 times greater than for DNA and deoxyadenylate-deoxythymidylate copolymer per mole of polymer. Its initial site of attack is 3'-hydroxyl end of the polydeoxyribonucleotide chain. Hydrolysis starting at the 5'-phosphoryl or 5'-hydroxyl end does not appear to occur with model oligonucleotides. The purified exonuclease II is closely associated physi-

cally with DNA polymerase and has not yet been dissociated from it. Like exonuclease I it has a pH optima in alkaline range. Lehman and Richardson[5] suggest according to the finding, that catalytic activity of E. coli exonuclease I and II is characterized exclusively by an exonucleolytic attack originating at the 3'-hydroxyl end of a polydeoxyribonucleotide, that this enzyme may find some use in current attempts to associate genetic markers with discrete positions on the DNA molecule. For example, by carrying out a stepwise degradation of biologically active DNA and by observing the sequential loss of selected markers, it is possible in principle to assign regions of recombination map, as determined by genetic crosses, to a region of DNA molecule in much the same way that Kaiser[73] has employed hydrodynamic shear to obtain correlations of this kind.

According to Lehman et al.[36,37] endonuclease I degrades native DNA at a 7-fold greater rate than thermally denatured DNA. It carried out an endonucleolytic attack on DNA yielding a limit digest whose oligonucleotides have an average chain length of approximately seven; these are terminated by 5'-phosphoryl groups. The isolated oligonucleotides are neither cleaved by high concentration of enzyme nor can they, when added in equal concentrations, inhibit the hydrolysis of DNA. It exists in the crude extract bound to an inhibitory RNA which is removed during purification. A unique feature of endonuclease I is its inhibition

by a variety of RNA from E. coli, guinea pig liver, and tobacco mosiac virus. Of those tested, RNA from E. coli is the most active. The kinetics of the enzyme inhibited by the RNA obey the equation of competitive inhibition, and the calculated K_i is of the order of 1×10^{-8} M (RNA nucleotide). It should be noted that, although tRNA and rRNA from E. coli are potent inhibitors of purified E. coli endonuclease, the possibility can not be excluded that there exist within the cell, other more potent RNAs whose specific function is, indeed in the immobilisation of this nuclease.

McCarty and Avery[76] observed potent DNase activity in the cell of Diplococcus pneumoniae (pneumococcus). Lacks and Greenberg isolated two nucleases with deoxyribonuclease activity from pneumococcus[40]. One of these, an endonuclease active on both native and denatured DNA, produced 5'-phosphate terminated fragments which even after extensive degradation were larger than mononucleotides. The other enzyme an exonuclease preferentially active on native DNA produced 5'-nucleotides and single stranded DNA. The exonuclease also exhibited a 3'-terminal phosphatase activity, it released 3'-terminal phosphate from 3'-terminated DNA. Exonuclease action generally levelled off when only 70% to 80% of the native DNA substrate had reacted. This presumably was due to the preexisting single strand breaks in the substrate which resulted in the formation of double stranded fragments isolated from further exonuclease action by dangling,

single stranded 3'-hydroxyl termini. Residual transforming activity in extensively degraded DNA may have corresponded to such persistent double stranded fragments. By attacking one strand of incoming DNA and thereby pulling the other strand into the cell, the exonuclease may play a role in the entry of the DNA in the genetic transformation of pneumococcus, since, immediately after entry, donar DNA appears to be equally divided between single strands and 5'-deoxynucleotides.

Little is known about the physiological role of bacterial nucleases. It has been proposed that pneumococcal DNases may function in the genetic transformation of pneumococcus, in the process of DNA entry[77] and recombination[78]. Furthermore, inactivation of transforming activity by DNases of known specificity offer a promising approach to the elucidation of the structures of transforming DNA and the mechanism of transformation.

Linn and Lehman[43] have described in the extracts of Neurospora crassa at least two physically separable nucleases at roughly comparable levels of activity in mycelia and conidia. One of these nucleases was purified 10,000 fold from the extracts of conidia and was found to be an endonuclease with a high degree of specificity for polyribo and polydeoxyribonucleotides lacking an ordered conformation. Denatured DNA and ribosomal RNA were hydrolysed at approximately the same rate. Both activities appeared to be catalysed by the same enzyme. The enzyme was shown

to be active under a variety of conditions including temperatures ranging from 25° to 65°, pH values from 6-9, the presence or absence of added divalent cations and salt concentrations ranging from 0.03 to greater than 0.24. The enzyme was inhibited by potassium phosphate and by EDTA. The inhibition by EDTA was reversed by stiochiometric amounts of Co^{2+} but not by large excess of Mg^{2+} ions.

The study of specificity and mode of attack on oligonucleotides and polynucleotides by Neurospora crassa enzyme[44] established that the purified enzyme preparation attacks native DNA at 2% the rate of denatured DNA. Hydrolysis of native DNA is due largely to the activity of contaminating nucleases which can be removed by a variety of treatments including incubation at 55° or exposure to thiols. Since the action of the enzyme was shown to be predominantly and may be exclusively endonucleolytic in character, the enzyme is capable of playing a role to remove single stranded regions from double stranded DNA, or to remove single stranded DNA, or RNA after DNA hybridization tests. The specificity of the enzyme makes this enzyme useful for the study of polynucleotide structure and conformation.

After the discovery of nucleases specific for single stranded DNA in E. coli by Lehman[27,79,80] and in the cells of E. coli infected with phage[81], pneumococcus and in snake venom[82], Ando described the presence of another nuclease in the products of

Aspergillus oryzae[45]. The nuclease specifically splitts the phosphodiester bond of heat denatured DNA. The enzyme has been purified 1,000 fold and demonstrated to be active in the presence of 10^{-4}M Zn^{3+} and less active in the presence of equal amounts of RNA. The digestion products from the denatured DNA have been identified as 5'-deoxymucleotides, thus resembling the action of *E. coli* exonuclease I. However, it differs with the latter in that it has an acid pH optima (4.4-4.6), and that this nuclease is inhibited by RNA. Study of the specificity of the S_1 nuclease of *Aspergillus oryzae* has been carried out by Wiegand *et al.*[33] According to them S_1 nuclease can digest single stranded DNA without introducing breaks in double stranded DNA. The enzyme is inhibited by low concentrations of phosphate. Under certain conditions S_1 nuclease cleaves the strand opposite a nick in bacteriophage T_5 DNA. Under other conditions the enzyme cleaves a loop in one strand of heteroduplex DNA while leaving the opposite strand intact. S_1 nuclease makes many single breaks in U.V. - irradiated duplex DNA. Superhelical DNA of ϕx174 (form I) is converted first to a relaxed circular molecule (form II) and then to a linear molecule (form III) by cleavage at one site per molecule. The utility of enzymes, having the kind of specificity reported for these enzymes, as selective reagents has been recognized in their wide-spread use. Still incompletely explored, however, is the use of these enzymes to

detect and characterize small variations in the secondary structure of nucleic acids. The kinds of variations in secondary structure, particularly those of biological significance are, for example the variations that would seem to be inherent in DNA that is being transcribed, replicated or recombined. Holloman et al.[84] have also described the action of S_1 nuclease on substrate which is akin to D-loops seen in replicating mitochondrial DNA, and which might also be an intermediate in genetic recombination. However, the use of these enzymes in the elimination of single stranded fractions after the DNA hybridization test has already been studied[85,86]. Also they have proven helpful for the study of single stranded DNA of coliphage ϕ x 174[87].

Shenk et al.[88] have reported a biochemical method using S_1 nuclease of Aspergillus oryzae to accurately map the locations of mutational alterations in Simian virus 40 DNA. Deletions of between 32-190 base pairs, which are at or below the limits of detectability by conventional electron microscope analysis of heteroduplex DNAs, have been located by this method. To map a deletion, a mixture of unit length, linear DNA prepared from SV40 deletion mutant and its wild type parent are denatured and reannealed to form heteroduplexes. S_1 nuclease can cut such heteroduplexes at the non base paired regions to produce fragments whose lengths correspond to the position of the deletion. Similarly specific fragments are produced when S_1 nuclease cleaves a

heteroduplex formed from DNAs of SV40 temperature sensitive mutants and either their revertants or wild type parents. Thus the position of the nonhomology between the DNAs can be determined.

A nuclease highly active on denatured DNA has been purified 9600 fold from the smut fungus Ustilago maydis[46]. The molecular weight of the enzyme is 42,000 as determined by gel filtration. Added divalent cations are not required for activity. EDTA strongly inhibits the activity, but simultaneous addition of the excess of Zn^{++} and Co^{++} reverses the inhibition. Other inhibitors include potassium phosphate, 2-mercaptoethanol, and ATP. The enzyme has a low level of activity upon native DNA. The U. maydis enzyme has been shown to liberate oligonucleotides from heat denatured DNA with a preferential attack at deoxyguanosine residues during the early course of hydrolysis[69]. However, after an exhaustive digestion, a large proportion of mononucleotides are found to accumulate. Examination of partial digests of denatured T7 DNA by successive gradient centrifugation indicate that DNA is reduced progressively to smaller fragments. Single stranded circular DNA from ϕ x 174, is refractory to hydrolysis by enzyme. This finding suggests that the enzyme requires DNA ends for activity. When DNA radioactively labelled specifically at 3'-end or 5'-end is digested with the nuclease, the label is liberated at a faster rate. The enzyme introduces single strand breaks in the linear duplex DNA. Certain mutant strains which are unable to carry out

allelic recombination have reduced amounts of this enzyme. Therefore, the enzyme playing some role in the recombination of the organism is a likely possibility.

Among the plant nucleases that specifically attack single stranded DNA, mung bean nuclease I reported by Sung and Laskowski[45] is so far the best studied enzyme. This nuclease has been purified extensively and the contaminating ribonuclease activity segregated by using sephadex G-100[89]. The enzyme shows optimal activity at pH 5.0 and has been classified as an endonuclease with a high degree of specificity towards single stranded DNA. Studies on the conformational specificity showed that calf thymus DNA was digested to the extent of 2.5 % whereas the same preparation was hydrolysed almost completely after thermal denaturation. This was interpreted (not necessarily correctly) as evidence that the preparation of native thymus DNA contained about 2.5 % of the denatured form, and that the enzyme has an absolute specificity for single stranded polynucleotides. Systematic investigation of the mung bean nuclease I on several species of DNA in their native, denatured and renatured states, showed that regardless of the substrate used (with the exception of $(dG)_n.(dC)_n$ which was resistant) some hydrolysis of native DNA occurred with high enzyme concentration[90]. The numerical value of the preference showed by mung bean nuclease I towards single stranded (denatured DNA) depends upon the type of DNA and the reaction conditions. At 37° in the

absence of Mg^{++} it is 30,000-fold for T4 DNA, 65-fold for $d(A-T)_n$, and less than 2-fold for biosynthetic $d(A-T)_n$. No dectectable hydrolysis of poly dG. poly dC occurs under the conditions even with a 100 fold excess of the enzyme. Addition of ad Mg^{++} inhibits the hydrolysis of biosynthetic $d(A-T)_n$ 3-fold but accelerates that of denatured DNA, 2 to 3-fold. The temperature coefficient of the reaction in the range 27-37° is 5-fold higher for biosynthetic $d(A-T)_n$ than for Crab $d(A-T)_n$. With native biosynthetic $d(A-T)_n$ conditions exist under which the rate of hydrolysis approaches that of denatured DNA from other sources (T4, E. coli, calf thymus). Therefore, mung bean nuclease I does not recognize biosynthetic $d(A-T)_n$ as a typically double stranded structure.DNA of phage λ which is known to contain an A-T rich region in the centre of the molecule is specifically attacked by the mung bean nuclease at this region.

In view of the studies carried out the important properties of mung bean nuclease I are, (a) its ability in low concentrations to remove denatured DNA from a mixture of both forms and (b) its ability in high concentrations to cleave specifically A-T rich regions of double stranded DNA. The name "region specific nuclease" was suggested for a new class of enzyme examplified by mung bean nuclease I. The mung bean nuclease has been used as a tool for the probe of low melting regions in DNA by Kedzierski and Laskowski[91]. Their results have shown that a highly purified

preparation of mung bean nuclease I removes the denatured regions from every species of DNA that was tested and the composition of these digested regions was richer in A and T than over all composition of DNA from which they were removed, regardless of the fact that the parent was A+T-rich or C+G-rich DNA. In the digested material C=G and A=T, therefore both strands were attacked simultaneously. The ability of mung bean nuclease as a good reagent to distinguish between the native and denatured forms has been recently shown on chinese hamster DNA by Papiero et al.[92].

Three different enzyme fractions degrading single stranded DNA have been fractionated from human aneuploid cell cultures[68]. One of these deoxyribonucleases DNase VI, has been thoroughly purified and characterized and its properties describe it to be the first novel enzyme in human or mammalian cells. The enzyme is clearly distinguishable from other known human DNases and also from those described in other mammalian cells. Thus DNase I is mainly extracellular, is not specific for single stranded DNA and has a neutral pH optima. DNase II is a lysosomal enzyme is not specific for single stranded DNA, has an acid pH optima and does not require Mg^{++}. DNase III is an exonuclease. DNase IV described in rabbit tissue[65] is an exonuclease specific for double stranded DNA. DNase V has a neutral pH optima and prefers double stranded DNA. The DNase specific for U.V.-irradiated, depurinated or Y-irradiated DNA[93-95] in human cells, as well as an alkaline

DNase described in human lymphocytes[96] do not require Mg^{++} for their activity. The DNase VI is considered to be the first broad spectrum DNase purified from mammalian cells after the Ca^{++} - Mg^{++} activated DNase[67]. The enzyme seems to correspond to an endonucleolytic activity previously observed in rat liver nuclei[97] and first recognized for its inhibiting action on a DNA polymerase preparation where it was a contaminant. DNase VI is an addition to the machinary available to the human cells for handling DNA. It is highly specific for single stranded DNA, since using the best preparations of DNA the rate of single strand break formation on native DNA is less than 0.25% of that on denatured DNA (less than 0.03% if judged by the lack of action on the nicked SV40 DNA). The enzyme probably recognized the limited areas of partial denaturation since it can act on supertwisted circular DNA though at a rate of the order of 0.1% of that on denatured DNA. The enzyme is an endonuclease that produces 3'-P and 3'-OH termini and requires Mg^{++} and an alkaline pH for its action.

The properties would make the enzyme a good candidate for removing small areas of local mispairing in normal DNAs , somewhat like the S_1 nuclease[69], and other similar nucleases from different organisms. The properties of DNase VI are analogous to those of the enzymes isolated from Neurospora crassa, Aspergillus oryzae, Ustilago maydis and Bacillus subtilis. These as described are highly specific for single stranded DNA and are capable of

attacking locally mismatched areas. An essential role of this sort of enzymes in the recombination process has been demonstrated for the *U. maydis* enzyme[89] and seems likely for *B. subtilis* one[98]. It is quite probable that DNase VI plays a similar role in human cells lines altered in DNA metabolism.

An endonuclease with a molecular weight of about 70,000 has been extensively purified from mouse ascites cells[52]. The enzyme specifically attacks single stranded DNA which is degraded mainly to oligonucleotides with 5-10 residues. Supercoiled covalently closed circular phage DNA is converted to the linear relaxed form. The enzyme activity is highly sensitive to salt and can be stimulated by reagents lowering the dielectric constant of the buffer such as dimethyl sulfoxide and glycerol. The enzyme shows a broad pH optima between 6.5 to 8.3, requires Mg^{++} for activity, whereas Ca^{++} inhibits the activity. Resembling this nuclease, another endonuclease appears in proliferating lymphocytes with the initiation of DNA synthesis phase of the cell cycle[99]. This finding suggests that the enzyme may be somehow involved in one of the reactions associated with DNA replication. It is as yet unknown which function in replication (if any) is performed by this endonuclease. The enzyme could for instance recognize unpaired regions or mismatched base pairs in the replicated DNA and may therefore have a vital proof-reading function. The enzyme can also attack tertiary twists in the DNA ahead of the replication

fork, thereby permitting a facilitated access of replication proteins to the template as well as the unwinding of DNA stem.

Another major endonuclease has been purified from rat liver nuclei using poly A as substrate[100]. The enzymatic activity was optimal between pH 6 and 7, and was totally dependent upon the presence of a divalent cation. The reaction was inhibited by high ionic strength, polydextran sulphate, heparin, and sodium pyrophosphate. The purified enzyme readily hydrolyses poly(A) poly(U), poly(C) and denatured DNA, whereas poly(G) was not degraded and transfer RNA, ribosomal RNA and native DNA were hydrolysed only at relatively slow rates. These data suggest that the enzyme may be specific for single stranded polynucleotides. The purified enzyme was essentially devoid of exonuclease activity and the products of exhaustive endonuclease digestion of poly(A) were small oligonucleotides terminated with a 5'-phosphoryl group. Evidence has been provided about the localization of the enzyme in the nucleoplasm. The nucleolar endonuclease and the nucleoplasmic exonuclease described by Lazarus and Sporn[101] appear to be the most abundant nuclease activities in nuclei and are the most likely candidate for the major salvage nucleases of heterogeneous nuclear RNA (hnRNA). The endonuclease releases products with 3'-hydroxyl end groups which exonuclease can use as substrate[102]. The major breakdown products of hnRNA are 5'-nucleotides[103]. The first specific cleavages of hnRNA processing may be an endonuclease

such as one specific for double stranded regions, but the discarded segments are probably degraded by these two enzymes which prefer single stranded polynucleotides. Even the reasonably large intranuclear turnover of the poly(A) regions of HnRNA may be attributable to these two nucleases. An activity similar to the endonuclease present in rat liver nuclei has been reported by Seppel[104] in pig liver nuclei, which too required a divalent cation for activity and releases 5'-oligonucleotides using poly(A) as substrate.

SCOPE OF THE PRESENT WORK

In the past several years much attention has been given to the study of nucleases that have a defined specificity. A large number of reports have appeared about enzymes that have an absolute specificity towards the degradation of single stranded DNA. Such nuclease activities have been shown to exist in bacteria and mammals including humans. During an initial study of nucleases that may have a role in DNA repair in plant systems an activity was encountered in germinating pea seeds that preferentially degraded single stranded DNA. The enzyme has been partially purified and characterized with respect to its physical properties and substrate specificity etc. Few reports are available about the existence of single stranded DNA specific nucleases in plants. The best studied

such nuclease from plants is that from mung bean sprouts[43].

The pea seed enzyme has been shown to degrade denatured DNA while the degradation of native double stranded DNA is quite low. The activity on native DNA can be further suppressed by the addition of salt. The enzyme is optimally active on pH 7.5 and shows activity at temperatures ranging from 30-70°. It seems to degrade low melting regions of DNA and is capable of degrading denatured and single stranded alkylated and depurinated DNAs. Since in the initial studies alkylated DNA was found to be preferentially degraded by the enzyme the effect of alkylation on the secondary structure was studied. Using hydroxyapatite chromatography it was shown that alkylation may lead to denaturation of the double stranded molecule under conditions where no significant depurination occurs. Some depurination of cellular DNA has been suggested to be taking place spontaneously under physiological conditions[105]. Since depurination may lead to partial denaturation the pea seed nuclease may quite possibly be acting at such regions to remove the damaged parts and thereby can have a role in DNA repair. In addition the enzyme can be used to study various features of DNA metabolism and can serve as a biochemical tool under a particular set of conditions where other nucleases fail to be of utility.

CHAPTER - II

MATERIALS AND METHODS

MATERIALS AND METHODS

MATERIALS

Biological material - Pea seeds (*Pisum sativum*) were obtained from the local market.

Chemicals - The following chemicals were obtained commercially and were used without further purification. Deoxyribonucleic acid (sodium salt) highly polymerized, average molecular weight 1 million; mercaptoethanol, crystalline bovine serum albumin, ovalbumin, α-chymotrypsinogen A, cytochrome C, 5',5'-dithiobis-(2 nitrobenzoic acid), reduced glutathione, pancreatic deoxyribonuclease were from Sigma Chemical Company (U.S.A.). EDTA, cysteine hydrochloride were purchased from B.D.H. Ltd.(England). Dimethyl sulfate was from May and Bakers Ltd. (England). Sephadex G-100, G-200 and Blue Dextan-2000 were obtained from Pharmacia Fine Chemicals (Sweden). DEAE-cellulose (Whatman DE-11) and Phosphocellulose (Whatman P-11) were purchased from W & R Balston Ltd. (England). Biogel A was obtained from Bio-Rad Laboratories, Richmond, California, U.S.A.

Hydroxyapatite was prepared according to the method of Bernardi[106].

Diphenylamine was obtained from B.D.H. Ltd. (England) and was recrystallized from boiling hexane.

The reagents used in polyacrylamide gel electrophoresis were: N,N,N',N', tetramethyl-ethyldiamine (Fluka, Switzeland); acrylamide, N,N'-methylene-bisacrylamide, amidoschwartz, and dichlorodimethyl silane (E. Merck, Germany), ammonium persulfate (Riedel De Haen AG, Germany); Bromophenol blue, glycerol, acetic acid, A.R. Grade (B.D.H., India).

All other chemicals used were of analytical reagent grade and were obtained from commercial sources. Glass double distilled water was used throughout the studies.

METHODS

Germination of seeds - The seeds were surface sterilized in 0.01% (W/V) mercuric chloride solution, and washed thoroughly with distilled water. The sterilized seeds were imbibed for 18 hours in sterilized distilled water at 25° or 4°. The seeds were then transferred to sterile petri dishes lined with moist filter paper. Sterilized distilled water was sprinkled after every 24 hours. The petri dishes were incubated at 25° for germination of seeds[107].

Crude enzyme preparation - The germinated seedlings were removed at the desired interval and the crude homogenate prepared

as follows. The seed coat was removed and the whole seedlings or the cotyledons or embryo axis separately were suspended in three volumes of potassium phosphate buffer (0.05M, pH 7.0) containing 1×10^{-3}M 2-mercaptoethanol. They were then thoroughly ground in a prechilled mortar or waring blender under cold conditions and the slurry passed through a cheese cloth. The filtrate was centrifuged at 1500 x g for 10 minutes and the supernatent obtained was used as the crude enzyme extract.

Preparation of denatured DNA - Denatured DNA was prepared by heating highly polymerized calf thymus DNA solution (2 mg/ml.) in TNE (0.01M Tris-HCl, 0.01M NaCl, 2×10^{-4}M EDTA, pH 7.5) at 100° for 6 minutes and rapidly cooling in ice[14]. Alkali denaturation of DNA was done by adding NaOH (2M) to a solution of DNA in TNE to a final concentration of 0.1M and keeping at room temperature for 5 minutes. The solution was neutralized to pH 7.5 by adding sufficient 1.1M HCl in 0.2M tris.

Preparation of depurinated DNA - Method for preparation of depurinated DNA was essentially that of Hadi and Goldthwait[14]. To a 5 ml solution of calf thymus DNA (2 mg/ml) in TNE, was added 6.5 ml of citrate buffer (0.1M, pH 3.5). The pH of the solution was thus decreased to 3.5. Depurination of DNA was usually done by heating this solution at 70° for 30 minutes and placing the tubes

in ice bath after this period. This was then neutralized to pH 6.5 by adding sufficient volume of 2M NaOH. Native DNA for use in the simultaneous assay was prepared by adding to the DNA solution an equivalent amount of citrate buffer neutralized to pH 6.5 with 2M NaOH. Thus the treatment of native DNA was similar to that of depurinated DNA except that it was not exposed to pH 3.5 and heat.

Alkylation of DNA and preparation of depurinated DNA from alkylated DNA - To 20 ml solution of DNA (2 mg/ml) in TNE was added 0.045 ml of 100% dimethyl sulfate (specific gravity 1.33) to give a DNA nucleotide to dimethyl sulfate molar ratio of 1:4. The mixture was shaken gently for 1 hour at room temperature. The pH was checked after every 5 minutes and any decrease in pH was corrected by addition of small volumes of concentrated NaOH. The alkylated DNA was divided into two halves. The first half was dialysed overnight against 50 volumes of TNE at 4°. Depurinated DNA was obtained by incubating the other half at 50° for 6 hours to achieve the release of labile alkylated bases[15]. The dimethyl sulfate and released alkylated bases were removed by overnight dialysis against 50 volumes of TNE at 4°.

Preparation of Cross Linked DNA - Cross linked DNA was prepared as described by Notani[109]. To 10 ml DNA (2 mg/ml) in TNE was added 10 ml of sodium nitrite (2M) in 0.5M sodium acetate

buffer, pH 4.5. The solution was kept at room temperature for 90 minutes and then chilled in ice. 5 ml of Na_2HPO_4 (2M) were added to bring the pH back to 6.5. The cross linked DNA thus formed was extensively dialysed against three changes (500 ml each) of TNE (Tris HCl 0.01M, NaCl 0.1M, EDTA 2×10^{-4}M, pH 7.5).

Enzyme assay - The reaction mixture in a final volume of 1 ml contained 0.35 ml of Tris-HCl buffer (0.2M, pH 7.5), 500 ug substrate (modified or unmodified DNAs), $MgCl_2$ to a final concentration of 1mM, 2-mercaptoethanol to a final concentration of 1mM, water and enzyme. The reaction mixture was incubated at 37° for the desired period of time. At the end of the incubation period, the reaction was stopped by the addition of 0.2 ml of 10 mg/ml bovine serum albumin solution (2 mg), mixed thoroughly by shaking and 1.2 ml of cold 14% perchloric acid. The tubes were left in ice bath for at least half an hour before centrifugation to remove precipitated protein and undigested DNA. The supernatant was analysed for acid soluble DNA-nucleotides.

Enzyme units and specific activity - One unit of enzyme was defined as the amount that causes the release of 1 umole of acid soluble DNA-nucleotide, per hour from substrate DNA under the standard conditions of assay. Specific activity was expressed as units per mg of protein.

Estimation of acid soluble nucleotides - DNA nucleotides, made acid soluble were determined by the diphenylamine method of Schneider[109] or spectrophotometrically by reading the absorbance at 260 nm. To a 2 ml aliquot, 2 ml of diphenylamine reagent (freshly prepared by dissolving 1 gm of crystalline diphenylamine in 100 ml of glacial acetic acid and 2.75 ml of conc. H_2SO_4) was added. The tubes were heated in boiling water bath for 20 minutes. The intensity of blue colour was read at 600 nm in Spectronic-20 Bausch and Lomb spectrophotometer. The amount of DNA made acid soluble was calculated from a standard curve. Adequate controls in which enzyme was added after the addition of PCA or those which contained only DNA were run in parallel. Results are expressed as umoles of DNA-nucleotide made acid soluble by the enzyme.

To determine the acid soluble material spectrophotometrically an aliquot of the supernatant was suitably diluted and read at 260 nm in Beckman model DU-spectrophotometer against a suitable blank.

Protein determination - Protein concentration was determined by the method of Christain and Warburg[110] and the method of Lowry et al.[111] using bovine serum albumin as standard. To a suitable aliquot of the crude homogenate, 3 ml of 10% (W/V) T.C.A. was added. The precipitate was centrifuged and washed successively three times with 3 ml of 10% T.C.A. The precipitate was dissolved in 5 ml of

0.1N NaOH. Protein in the subsequent purification steps could not be precipitated with TCA, and therefore an aliquot was taken directly. A suitable aliquot of protein solution was diluted to 1 ml with water. To this was added 5 ml of freshly prepared copper reagent (by mixing 3.5% $CuSO_4$ in 1% (W/V) potassium tartarate and 2% (W/V) Na_2CO_3 in 0.1N NaOH, in 1:50 ratio). After incubation for 10 minutes at room temperature, 0.5 ml of Folin-ciocalteau reagent (1N) was added and instantly mixed. The colour intensity was read after 30 minutes against reagent blank in Spectronic-20 Bausch and Lomb spectrophotometer at 660 nm.

Column Chromatography

Sephadex Column - Analytical column of sephadex G-200 for the determination of molecular weight of the enzyme, was prepared according to the standard procedure supplied by the Pharmacia Fine Chemicals, Sweden. A required amount of sephadex G-200 was allowed to swell in a suitable amount of double distilled water for 6 hours in boiling water bath. A glass column previously cleaned with chromic acid, detergent and distilled water was mounted vertically. One third of the column length was filled with the operating buffer and then slurry of the gel (deaerated under vaccum) was poured into it with the help of a glass rod. The column was left standing for about one hour till the gel had settled to some extent. During this period, the stopcock was opened with slowly increasing rate.

After accomplishing the constant rate of flow, higher than required final elution, rate was adjusted to normal. The column was thoroughly washed with a volume of operating buffer (3-4 times the bed volume) to ensure no further sarinking of the bed height during the experiment. To determine the uniform packing and to determine the void volume (Vo) of the column 0.2 percent solution of Blue Dextran was passed through the column. The volume of the dye was 3 percent (or lower) of the total bed volume of the column. The buffer was then removed from the gel surface and protein solution (2-3 percent of the total bed volume) was carefully applied.

The column of Sephadex G-100 and Biogel-A were also prepared in a similar manner.

DEAE-Cellulose Column - Fifteen gm of DEAE-Cellulose was suspended in a suitable amount of distilled water. The exchanger was then treated with HCl (0.5N final concentration) for half an hour and washed in a buchner funnel with distilled water. The treatment was repeated and washing was continued till the pH of filtrate was approximately 4.0. The exchanger was then treated with NaOH (0.5N final concentration) for half an hour. The treatment with alkali was repeated and washing was continued till the pH approached neutrality. The DEAE-cellulose cake was suspended in the operating buffer and was stirred gently to achieve a homo-

genous slurry. The fine particles were removed from the slurry, before applying to a clean vertically mounted column, with the help of a glass rod. After about an hour when the exchanger had settled, the stop-cock was opened and the flow rate was gradually increased to the desired final flow rate. The column was then equilibrated with a large excess of the buffer, till the pH of the effluent was identical to that of the eluting buffer. The column was connected to the gradient assembly consisting of two gradient vessels. The mixing vessel contained 0.02M tris-HCl buffer, pH 8.0. The salt concentration in successive fractions was calculated using the following equation[112].

$$C = C_2-(C_2-C_1)_e-V/V0$$

Where C is the salt concentration of the protein fraction, C_1 is the salt concentration of the buffer in the mixing vessel, C_2 is the salt concentration of the buffer in the second vessel connected to the mixing vessel, V is the volume in each fraction and Vo is the volume in the mixing vessel which was maintained at 300 ml in our case.

Phosphocellulose column - Fifteen gm of phosphocellulose was suspended in distilled water and treated with NaOH (final concentration 0.5N) for half an hour and washed on a buchner funnel. The treatment was repeated and the exchanger washed till the pH was approximately 9.0. The cake was then suspended in 0.5N HCl

for half an hour and washed as above. The treatment was repeated and the washing was done till all the acid was removed. The exchanger was suspended in the equilibrating buffer and stirred gently to achieve a homogeneous slurry. The column of phospho-cellulose was prepared in a way similar to DEAE-cellulose column, with the exception that the equilibrating buffer in this case was phosphate buffer (0.02M, pH 6.5). The mixing chamber initially contained equilibrating buffer while the other vessel contained 0.8M NaCl in the same buffer.

Hydroxyapatite Column - Hydroxyapatite was suspended in phosphate buffer (0.01M) containing 1% formaldehyde. The fine particles were removed and the slurry mainly containing course particles was poured into a column of 1 cm cross section. The stop-cock was opened and sufficient amount of fluid was allowed to pass to obtain a 3 cm bed. DNA samples were applied and the elution was carried out with a stepwise gradient of phosphate buffer containing 1% formaldehyde.

Polyacrylamide Disc Gel Electrophoresis - Polyacrylamide disc gel electrophoresis was performed essentially according to the method of Davis[113] using 7.5% acrylamide gel. Glass tubes of uniform diameter were held vertically with lower ends closed by rubber stoppers. To each tube was added about 2.0 ml of the small

pore gel. The tubes were allowed to stand for 30 minutes at room temperature, till the gel polymerized. Sample and stacking gels were omitted. A suitable aliquot of enzyme sample (whose density was increased with 20% Glycerol) was applied to the surface of the gels. Electrophoresis was carried out for 2 hours using a current of 3mA/tube untill the dye, bromophenol blue reached the bottom of the gel. The electrode buffer was Tris-Glycine 0.01M pH 8.3. After the electrophoresis the gels were removed from the tubes by rimming the sides of the tubes with a long thin needle, and were stained with 1 percent Amido Schwarts in 7% (V/V) acetic acid for 30 minutes. The gels were destained through repeated changes of 7% (V/V) acetic acid.

CHAPTER - III

R E S U L T S

Figure - 1. Effect of crude extract from "72 hour" germinated seedlings on normal and depurinated DNA - The reaction mixture in 1 ml contained 400 ug of DNA (native and depurinated) 1 mM $MgCl_2$, 1 mM 2-mercapto-ethanol, 0.2 ml SSC (0.1M NaCl, 0.05M sodium citrate, pH 7.0) and 0.95 mg of crude enzyme protein. The pH was 7.0. Incubation was carried out at 37° and aliquots of 1 ml were removed at the time intervals indicated and reaction stopped as described in the methods. Acid soluble nucleotides released by the enzyme were estimated. In order to determine spontaneous release of acid soluble material from depurinated DNA a control experiment with this DNA lacking enzyme was also run in parallel.

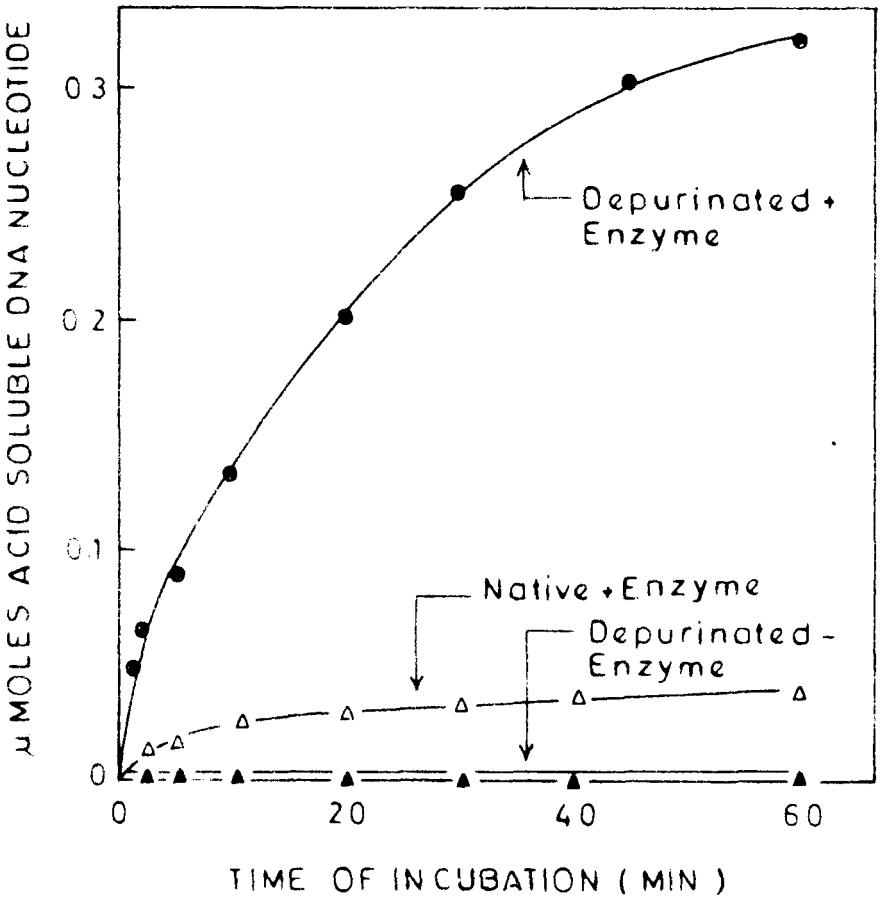

μ MOLES ACID SOLUBLE DNA NUCLEOTIDE
0.3
0.2
0.1
0
0
20
40
60
TIME OF INCUBATION (MIN)
Depurinated + Enzyme
Native + Enzyme
Depurinated - Enzyme

Figure - 2. Effect of crude extract upon native, alkylated and depurinated DNA - Reaction mixture in 1 ml contained 300 ug of substrate, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 0.35 mg of enzyme protein. The reaction was done and stopped as described in methods and nucleotides made acid soluble estimated.

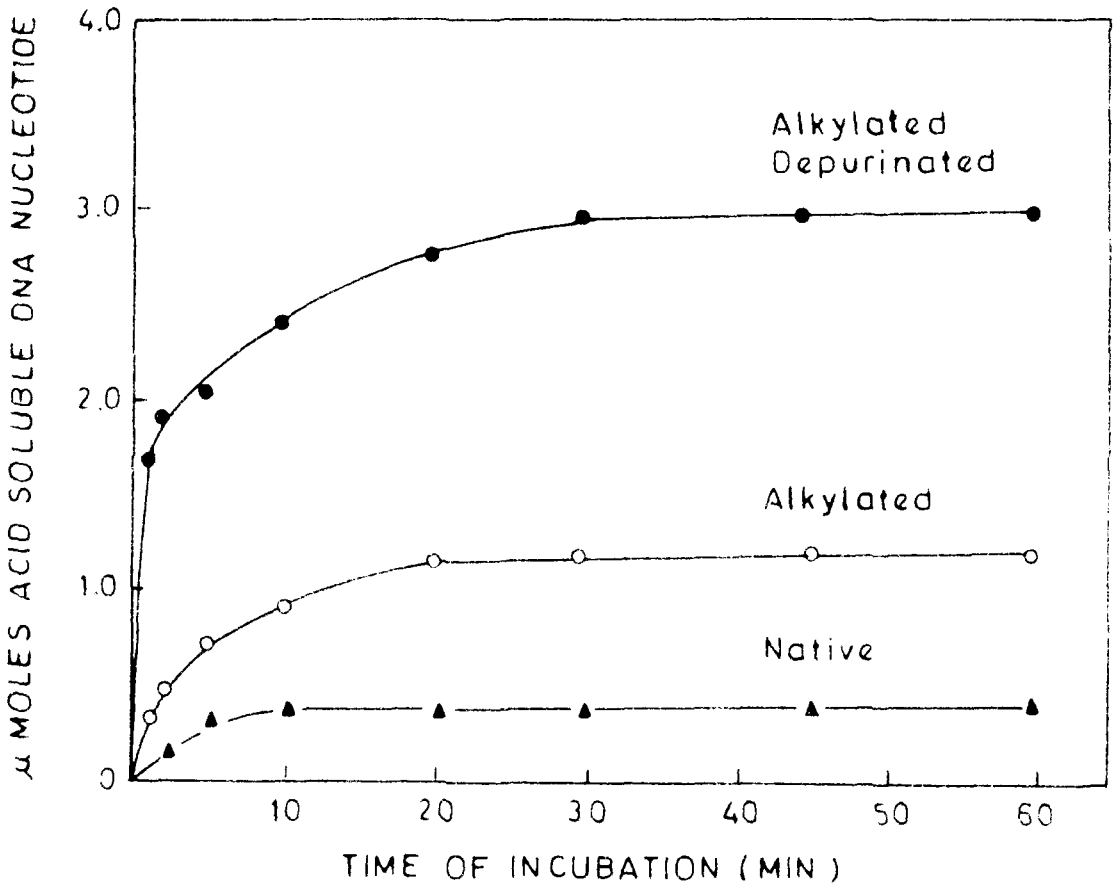
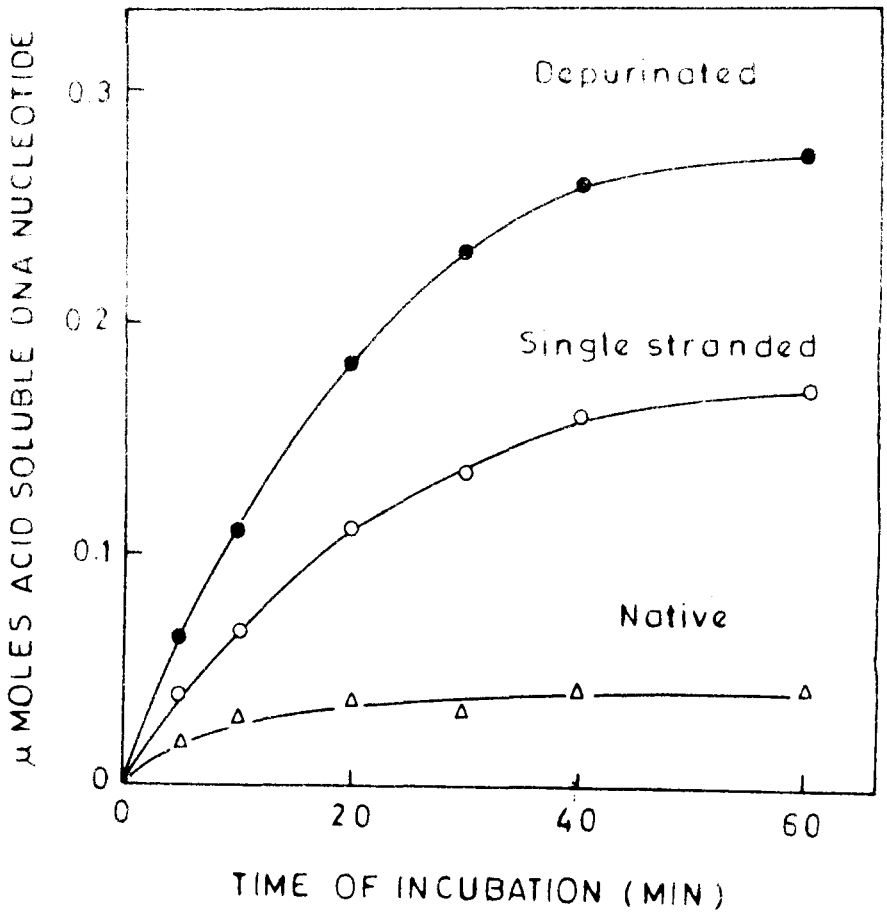

μ MOLES ACID SOLUBLE DNA NUCLEOTIDE
4.0
3.0
2.0
1.0
0
Alkylated
Depurinated
Alkylated
Native
10
20
30
40
50
60
TIME OF INCUBATION (MIN)

Figure - 3. <u>Effect of crude extract from "72 hour" germinated axis upon native, denatured and depurinated DNA</u> - Depurinated DNA was prepared by heating DNA at 70° for 40 minutes at pH 3.5. Denaturation of DNA was done by heating at 100° for 7 minutes and rapidly cooling in ice. Reaction mixture in 1 ml contained 500 ug of DNA (native, denatured and depurinated) 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.2 ml SSC and 0.5 mg of crude enzyme protein. The pH was 7.0. Incubation was carried out at 37° and aliquotes of 1 ml were removed at time intervals indicated and reaction stopped as described in the methods. Acid soluble nucleotides released by the enzyme were estimated.


Depurinated
Single stranded
Native
0.3
0.2
0.1
0
0
20
40
60
μ MOLES ACID SOLUBLE DNA NUCLEOTIDE
TIME OF INCUBATION (MIN)

Effect of crude extract on native, alkylated and depurinated DNA

Depurination of DNA was done using a milder technique. DNA was first alkylated with dimethyl sulfate at pH 7.0 and then heated at 50° for several hours at pH 7.0 in order to release alkylated purines. This resulted in the appearance of apurinic sites as shown by alkaline hydrolysis of such DNA. Fig. 2 shows the degradation of native, alkylated and depurinated DNA obtained by release of alkylated purines. As is evident, the production of maximum acid soluble material from depurinated DNA was significantly higher than that from native DNA. Some degradation of alkylated DNA above that of native DNA was also observed. This preferential hydrolysis of alkylated DNA could not be explained at the moment. However, as will be shown later it was determined to be due to single stranding of DNA on alkylation.

Effect of crude extract on native, denatured and depurinated DNA

Fig. 3 shows the comparative degradation by crude extract, of native, denatured and depurinated DNA as a function of time. As observed earlier the maximum degradation of native DNA was low. However, it was considerably higher in case of denatured DNA. Further, the depurinated DNA was hydrolysed to an even greater extent than the denatured substrate. These observations along with the results presented in Fig. 1 and 2 suggested the presence in crude extract of one or more nucleases that preferred single stranded DNA either in alkylated or depurinated form.

Effect of germination time on the degradation of native and denatured DNA

The activity of nuclease at different stages of germination of seeds was studied. Experiments with crude extract from the cotyledons as well as from embryo axis were carried out. Specific activity of degradation on native as well as denatured DNA in these organs at different stages of germination was determined. The specific activity with denatured DNA was again found higher. It is clear from the results obtained in Table I, that the enzyme was present even in the seeds imbibed at 4° and dry seeds. Some increase in specific activity over a 120 hr germination period was observed both in cotyledons as well as embryo axis.

Purification of pea seed nuclease specific for single stranded DNA

The enzyme was purified from embryo axis of germinated seeds. Pea seeds were germinated in sterile trays lined with moist filter sheets at 25°. After 76 hour germination seeds were removed and embryo axis excised. Unless otherwise mentioned all steps were carried out at $0-4^{\circ}$.

Step - 1: Preparation of Crude Extract - 200 gm Embryo axis were homogenized in a prechilled waring blender with cold 300 ml buffer A (Tris-HCl 0.02M, pH 8.0 containing 1×10^{-3}M mercapto-ethanol). The homogenate was passed through folds of cheese cloth

TABLE - 1

EFFECT OF GERMINATING PERIOD ON THE NATIVE AND DENATURED DNA DEGRADING ACTIVITY OF CRUDE EXTRACTS OF COTYLEDONS AND EMBRYO AXIS.

The specific activities were calculated using linear enzyme concentration versus activity plots. It is assumed that the degradation of single stranded DNA by crude extract include the action of non-specific nucleases. Degradation of native DNA by a given amount of enzyme was therefore subtracted from the degradation of single stranded DNA before calculating the units of single stranded DNA specific nuclease.

GERMINATION PERIOD	SPECIFIC ACTIVITY			
	DENATURED DNA		NATIVE DNA	
	Cotyledons	Embryo axis	Cotyledons	Embryo axis
Dry Seeds	0.033	-	0.004	-
Imbibition				
18 hr, 4°	0.047	0.059	0.006	0.002
18 hr, 25°	0.051	0.060	0.007	0.012
Germination				
24 hr, 25°	0.054	0.092	0.008	0.019
72 hr, 25°	0.055	0.100	0.008	0.021
120 hr, 25°	0.058	0.119	0.009	0.022

and centrifuged at 1500 x g for 20 minutes in cold to remove cellular debris (Fraction I).

Step - 2: Ammonium sulfate fractionation - To 350 ml of fraction I, 79.1 gm of solid ammonium sulfate was added and dissolved by stirring (40% saturation). The pH was maintained at 8.0 by intermittent addition of NH_4OH. After 60 minutes, the precipitate was removed by centrifugation at 1500 x g for 20 minutes. The precipitate was dissolved in 50 ml of buffer A (0-40% fraction). To the supernatant solution an additional 53.5 gm of solid ammonium sulfate was added (65% saturation) and dissolved slowly by stirring. The solution was allowed to stand in cold for 2 hours till the precipitate settled. The precipitate was recovered by centrifugation at 1500 x g for 30 minutes and was dissolved in 60 ml of buffer A (40-65% fraction). To the supernatant was added further 33.9 gm ammonium sulfate (80% saturation) with gentle stirring and the precipitate allowed to settle in cold for 2 hours. The resulting precipitate was recovered by centrifugation at 1500 x g for 20 minutes and dissolved in 40 ml of buffer A (65-85% fraction). The supernatant which contained only negligible nuclease activity was discarded. All the three fractions were dialysed extensively against four changes of buffer A (1 litre each) till all ammonium sulfate was removed. As shown in Table 2, the activity for denatured DNA is present in both 40-65 and 65-80% fractions. The

TABLE - 2

AMMONIUM SULFATE FRACTIONATION

See text for details

FRACTION	SPECIFIC ACTIVITY	
	DENATURED DNA	NATIVE DNA
CRUDE	0.54	0.037
0 - 40%	0.36	0.033
40 - 60%	1.20	0.044
65 - 80%	1.03	0.031

1 unit of enzyme is the amount of enzyme protein that causes the liberation of 1 umole of acid soluble DNA nucleotide in 1 hr at 37° under standard assay conditions.

Specific activity is units per mg protein.

Figure - 4. Chromatography on DEAE-cellulose - The column (1.6 x 35 cm) was equilibrated with 1.5 litres of 0.2M Tris-HCl buffer, pH 8.0. 270 mg of protein (fraction II) in the equilibrating buffer was applied to the column. The column was washed with 200 ml of the same buffer before elution. 4 ml fractions were collected using a linear salt gradient of 0-0.5M at a flow rate of 30 ml/hr.

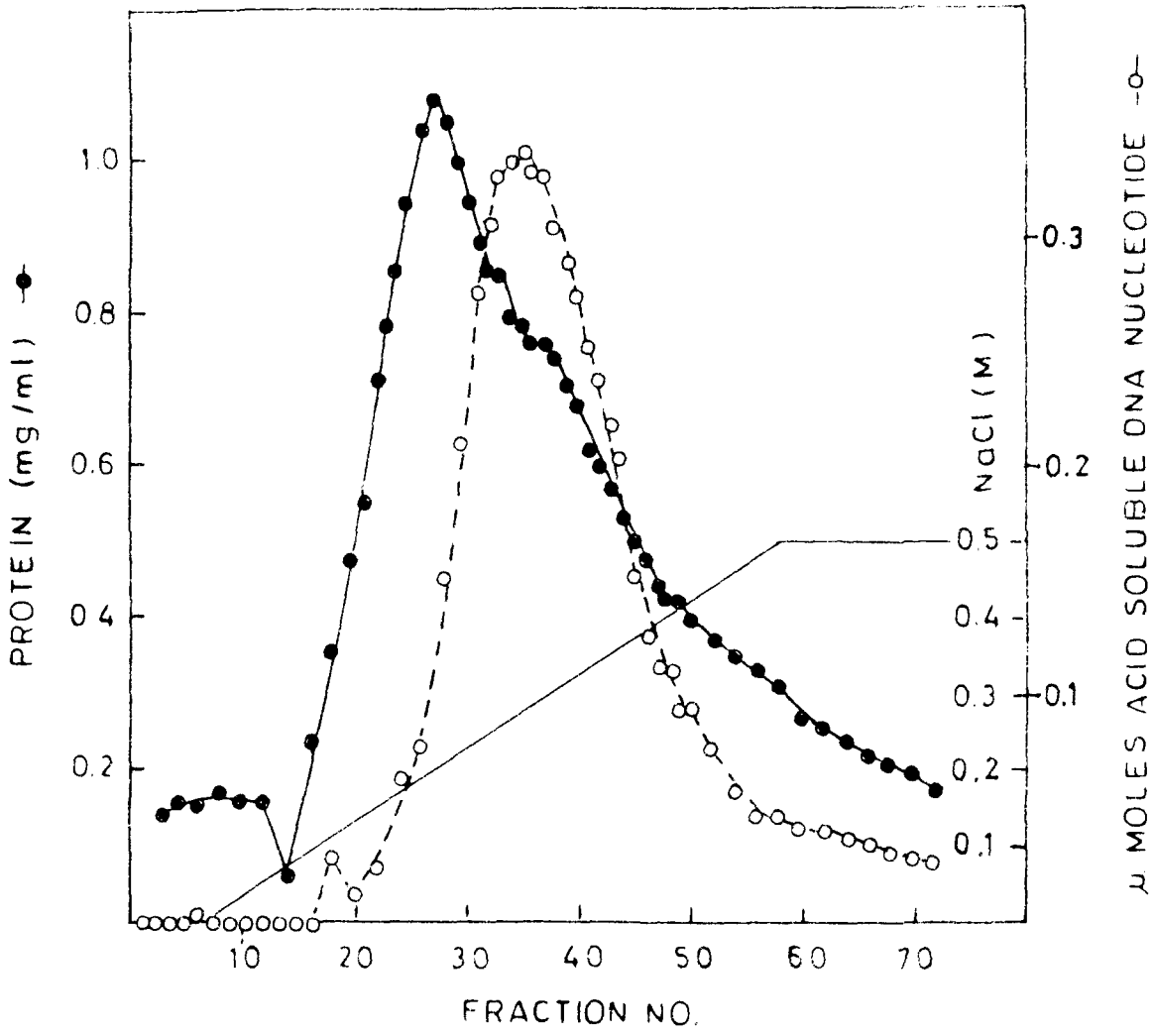
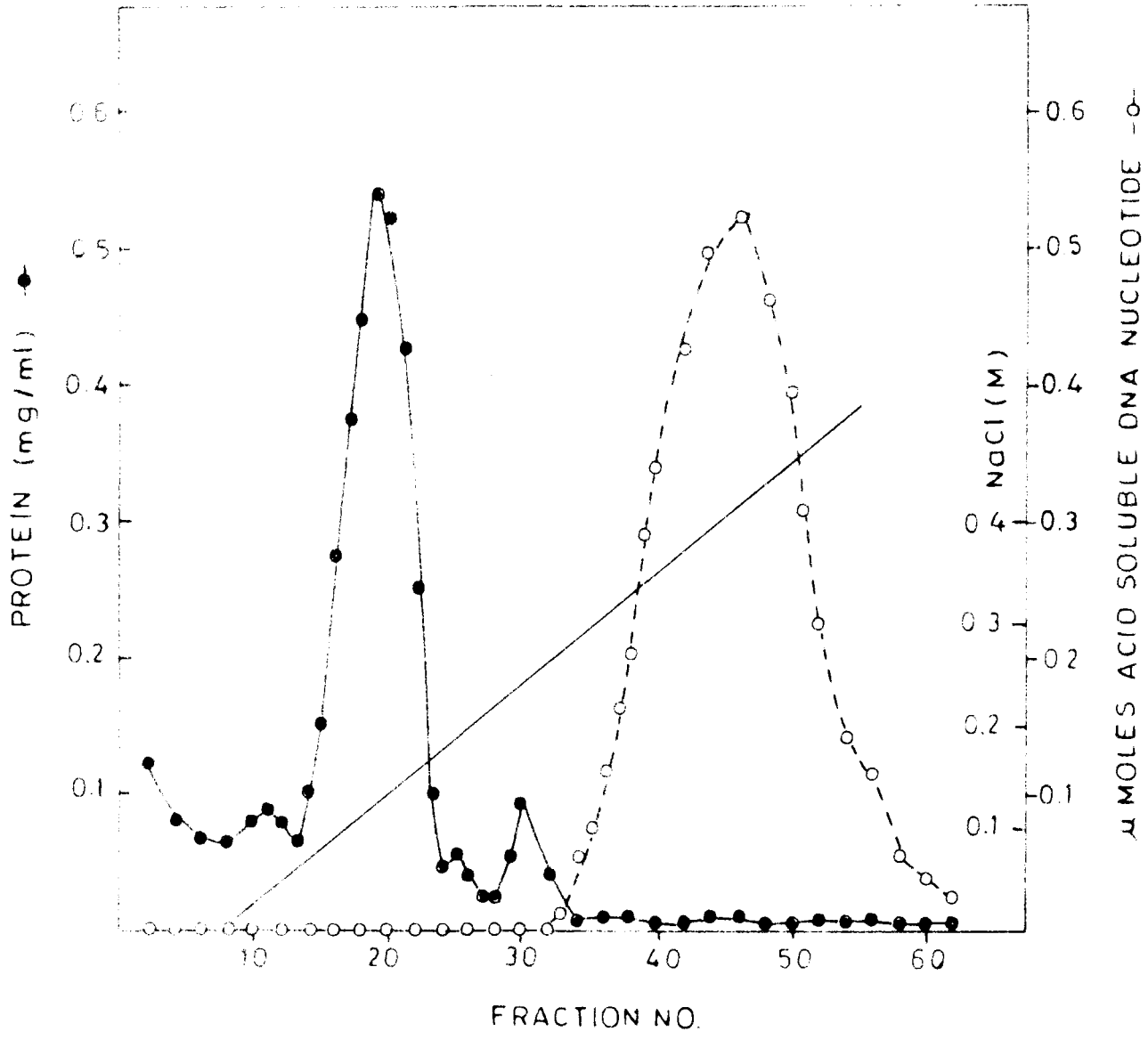

PROTEIN (mg/ml)
1.0
0.8
0.6
0.4
0.2
NaCl (M)
0.5
0.4
0.3
0.2
0.1
0.3
0.2
0.1
μ MOLES ACID SOLUBLE DNA NUCLEOTIDE
10
20
30
40
50
60
70
FRACTION NO.

Figure - 5. Chromatography on Phosphocellulose - The column (1.6 x 25 cm) was equilibrated with 1.5 litres of 0.02M phosphate buffer pH 6.5. 50 mg of protein (fraction III) in the equilibrating buffer was applied to the column. The column was washed with 300 ml of the same buffer. Elution was performed with a linear salt gradient of 0–0.4M at a flow rate of 30 ml/hr. 4 ml fractions were collected.


PROTEIN (mg/ml)
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
FRACTION NO.
NaCl (M)
0.4
0.3
0.2
0.1
μ MOLES ACID SOLUBLE DNA NUCLEOTIDE

two fractions were mixed (Fraction II) before further purification.

Step - 3: Chromatography on DEAE-cellulose - The dialysed fraction II (270 mg protein) was applied to a column of DEAE-cellulose (1.6 x 35 cm) equilibrated with buffer A. After allowing the flow through to pass, the column was washed with 200 ml of the equilibrating buffer. A linear gradient (300 ml) of 0-0.5M NaCl in buffer A was applied to elute the enzyme. Pea seed nuclease activity appeared as a broad peak between 0.2-0.4M NaCl. In several repeated experiments the chromatographic profile of Fig. 4 was found reproducible. The active fractions 28-50 (30 ml) were pooled and dialysed against buffer B (potassium phosphate 0.02M, pH 6.5, containing 1 x 10^{-3}M mercaptoethanol) for 20 hours in cold (Fraction III).

Step - 4: Chromatography on Phosphocellulose - Fraction III (80 mg protein) was applied to a column of phosphocellulose (1.6 x 20 cm) equilibrated with buffer B. The column was washed with 200 ml of equilibrating buffer and a linear gradient (200 ml) of 0-0.4M NaCl in the same buffer was applied to elute the enzyme. The activity of the enzyme appeared as a single peak at 0.4M NaCl after the bulk of protein had eluted. The chromatographic profile of Fig. 5 was reproducible in several experiments. The active fractions 34-36 (90 ml) were pooled and concentrated on dry sucrose. The concentrated enzyme was dialysed against several

Figure - 6. Disc gel electrophoresis of pea seed nuclease - 7.5% gels with Tris-glycine buffer system pH 8.3 were used. Pattern is shown for purified enzyme obtained from phosphocellulose column (fraction IV). Migration took place from top (-) to bottom (+). See text for details.

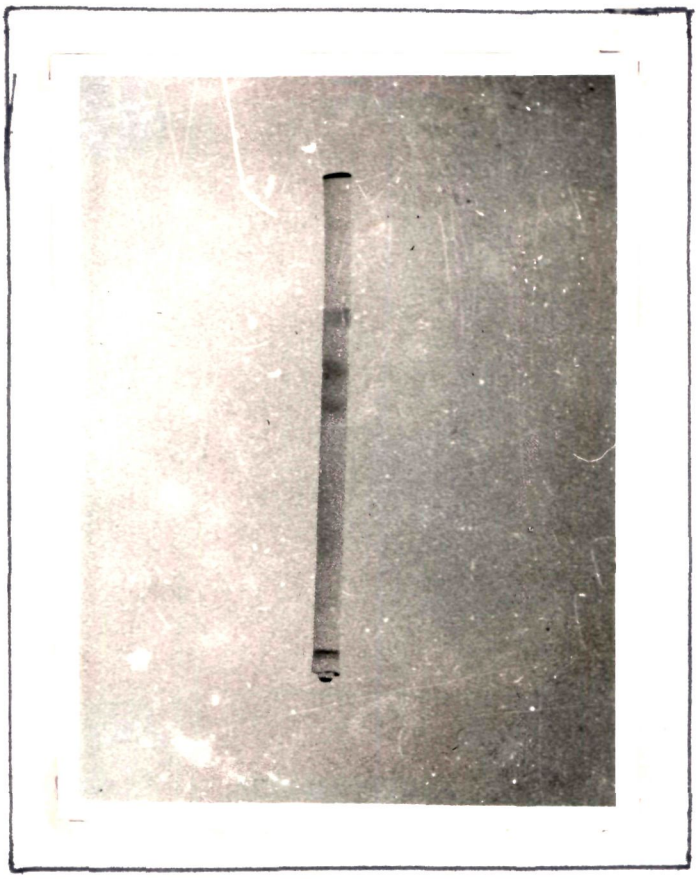


TABLE - 3

SUMMARY OF PURIFICATION OF NUCLEASE FROM PEA SEED

FRACTION	VOLUME (ml)	PROTEIN (mg/ml)	TOTAL ACTIVITY (units)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (units/mg protein)	RECOVERY (%)	FOLD PURIFICATION
CRUDE HOMOGENATE	950	4.08	1705	3876	0.44	100	1.00
AMMONIUM SULFATE (40-80% cut)	194	5.63	1375	1092	1.26	80	2.86
DEAE-CELLULOSE	110	1.63	1045	179.3	5.83	61	13.25
PHOSPHOCELLULOSE	29	0.20	360	5.8	62.11	21	141.15

changes of 0.02M Tris-HCl buffer, pH 7.5 containing 1×10^{-3}M mercaptoethanol. The enzyme was frozen in the same buffer with 10% glycerol, in several small aliquots (Fraction IV).

Fraction IV was stored at -5° and retained unchanged activity for 9 months. Thawing and refreezing caused small losses of activity (5 to 10%). Table 2 summarizes the percent yield, specific activity and the extent of purification of the enzyme preparation at each step of purification.

On polyacrylamide disc gel electrophoresis using 7.5% acrylamide at pH 8.3, fraction III gave a number of bands, but fraction IV showed only three discrete bands as represented in Fig. 6. Due to the appreciable yield and high specific activity detailed physico-chemical studies were done with fraction IV.

Effect of increasing concentration of enzyme on native, denatured and depurinated DNA

Denatured DNA (alkali and heat denatured) was incubated with increasing concentrations of fraction IV. There was a linear release of acid soluble DNA nucleotides from either substrate. The rates of hydrolysis of both alkali and heat denatured DNAs were similar. However there was little degradation of native DNA under similar conditions (Fig. 7). In the experiment shown in Fig. 8 the enzymatic degradation of denatured DNA and DNA depurinated by heating at pH 3.5 was compared. The enzyme degraded both

Figure - 7. Effect of enzyme concentration on native, heat and alkali denatured DNA - Reaction mixture in 1 ml contained 500 ug substrate, 0.05M tris-HCl, pH 7.5, 1mM $MgCl_2$ and increasing amounts of enzyme (Fraction IV, sp. act. 62). The incubation was done for 1 hr. at 37°, and reaction processed as described earlier.

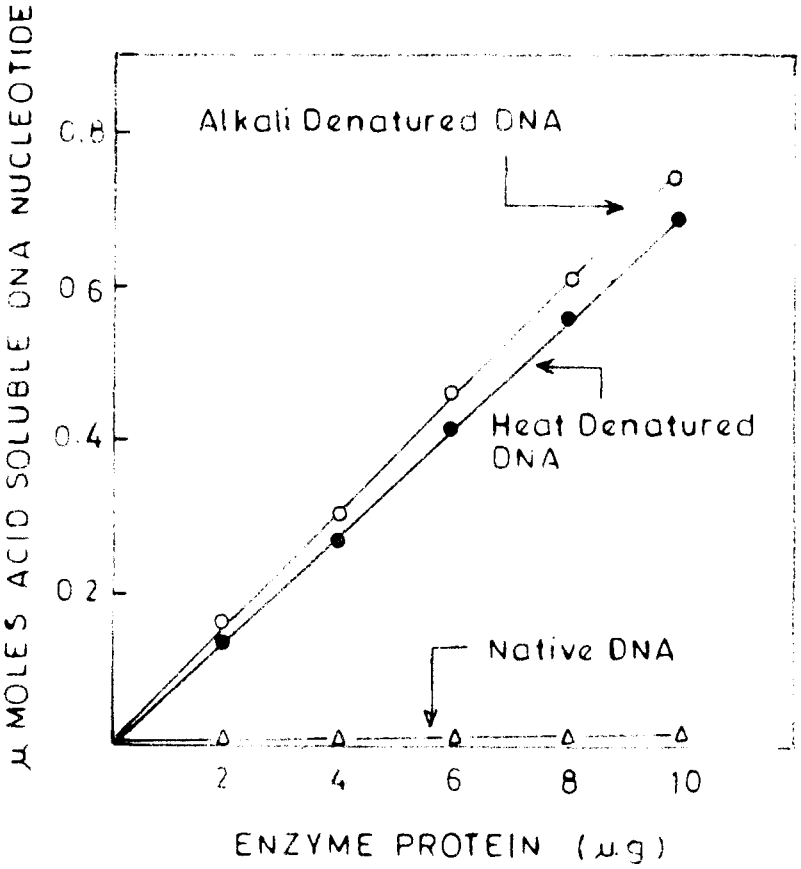
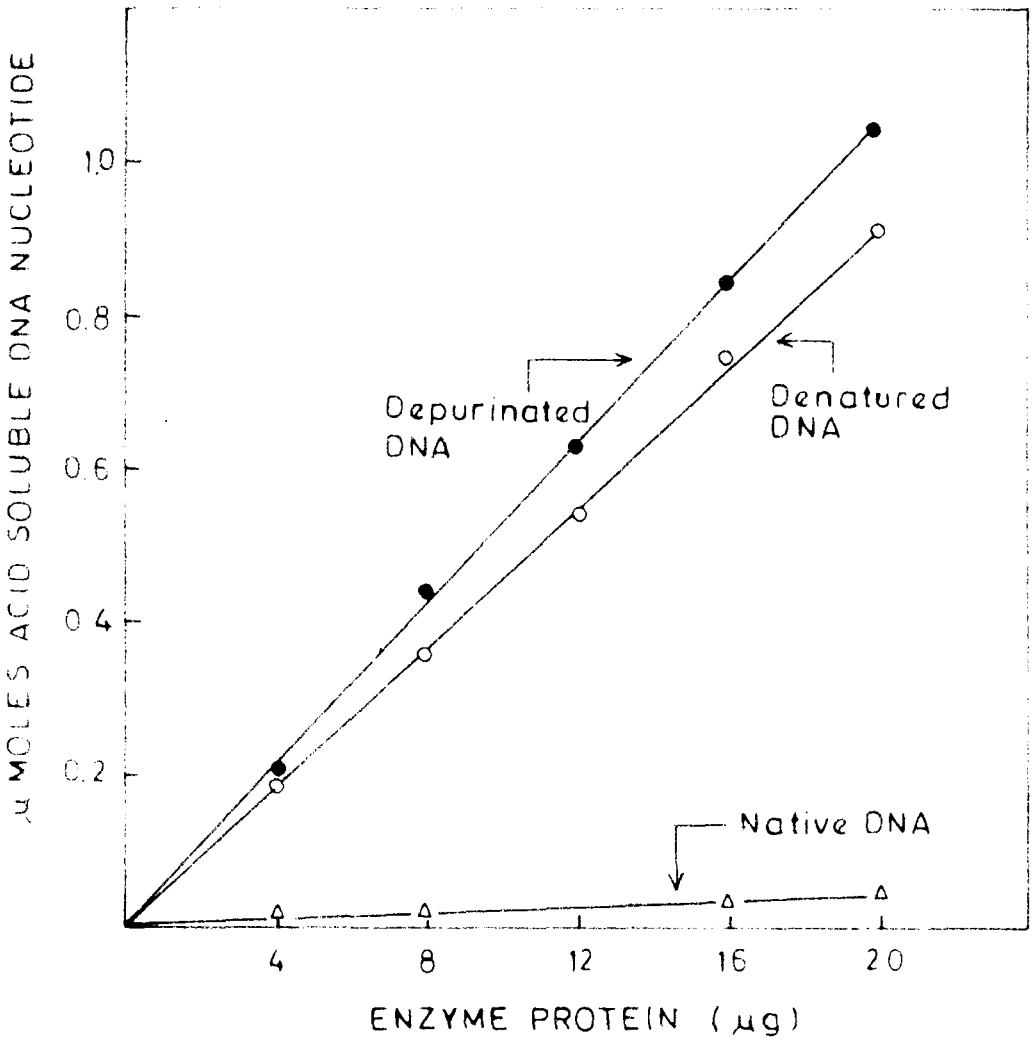

μ MOLES ACID SOLUBLE DNA NUCLEOTIDE
0.8
0.6
0.4
0.2
Alkali Denatured DNA
Heat Denatured DNA
Native DNA
2
4
6
8
10
ENZYME PROTEIN (μg)

Figure - 3. Enzymatic degradation of native, denatured and depurinated DNA - The reaction mixture in 1 ml contained 300 ug DNA (native, single stranded or depurinated) 0.2 ml SSC (0.1M NaCl, 0.05M sodium citrate, pH 7.0), 1 mM $MgCl_2$ and enzyme (Fraction IV, sp. act. 62) in indicated concentrations. The incubation was done at 37° for 1 hr. and DNA nucleotide acid solubilized by enzyme was determined as described.


μ MOLES ACID SOLUBLE DNA NUCLEOTIDE
1.0
0.8
0.6
0.4
0.2
Depurinated DNA
Denatured DNA
Native DNA
4
8
12
16
20
ENZYME PROTEIN (μg)

denatured and depurinated DNA with almost equal rates. It should be recalled here that depurination under the conditions carried out resulted in denaturation of DNA[14]. Degradation of native DNA was again found to be low. These results indicated that the purified pea seed nuclease may be specific for single stranded DNA. The results described earlier with the crude enzyme extract (Fig.3) showed greater degradation of depurinated DNA than single stranded DNA. But with purified enzyme the two activities were at comparable levels. It seems possible that the crude extract contained in addition to the enzyme specific for denatured DNA another enzyme that may prefer depurinated DNA and which was removed during the process of purification.

Effect of time of incubation on nuclease activity

Fig. 9 shows the enzymatic hydrolysis of denatured and native DNA as a function of time. Hydrolysis of denatured DNA reached a plateau at 60 minutes when 100% of the substrate was rendered acid soluble. In contrast the hydrolysis of native DNA did not proceed beyond 7% of the substrate and this was also completed in 60 minutes. The plateau obtained was not in either case due to inactivation of the nuclease since a further addition of substrate after 2 hours resulted in a further spurt of activity. In the case of native DNA the reaction stopped again after the hydrolysis of further 7% of DNA was achieved. This indicated that

Figure - 9. Time course of digestion of native and denatured DNA - Alkali denatured DNA was used in the experiment. The reaction mixture in 1 ml contained 150 ug denatured DNA, 0.05M Tris-HCl buffer, pH 7.5, 1 mM $MgCl_2$ and 0.6 units of enzyme (Fraction IV, sp. act. 62). The native DNA reaction mixture contained 1 mg of substrate and 0.1M NaCl. The incubation was done at 37° and 1 ml aliquots were removed at indicated times and processed by the standard procedure. After 2 hr. an equivalent amount of additional substrate was added to the incubation mixture and reaction followed as before.

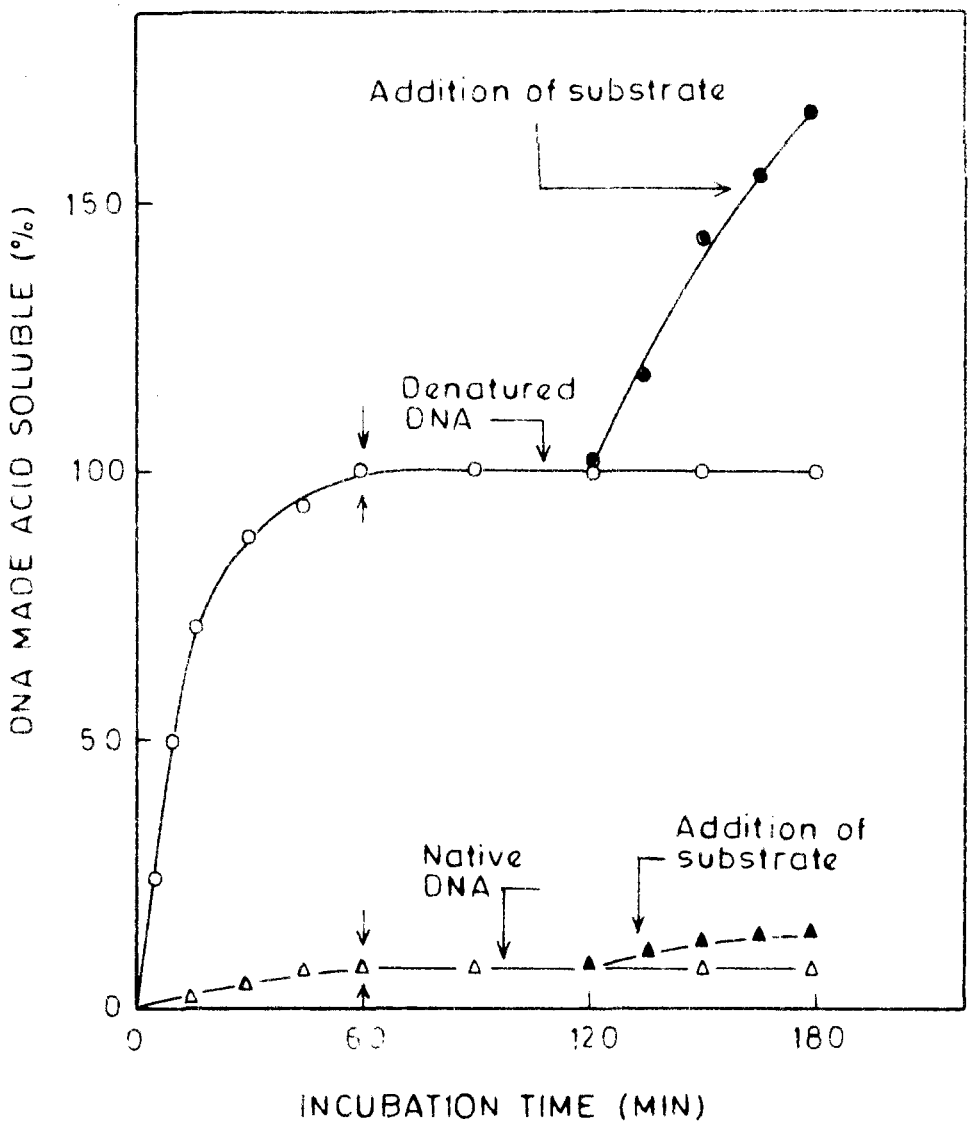
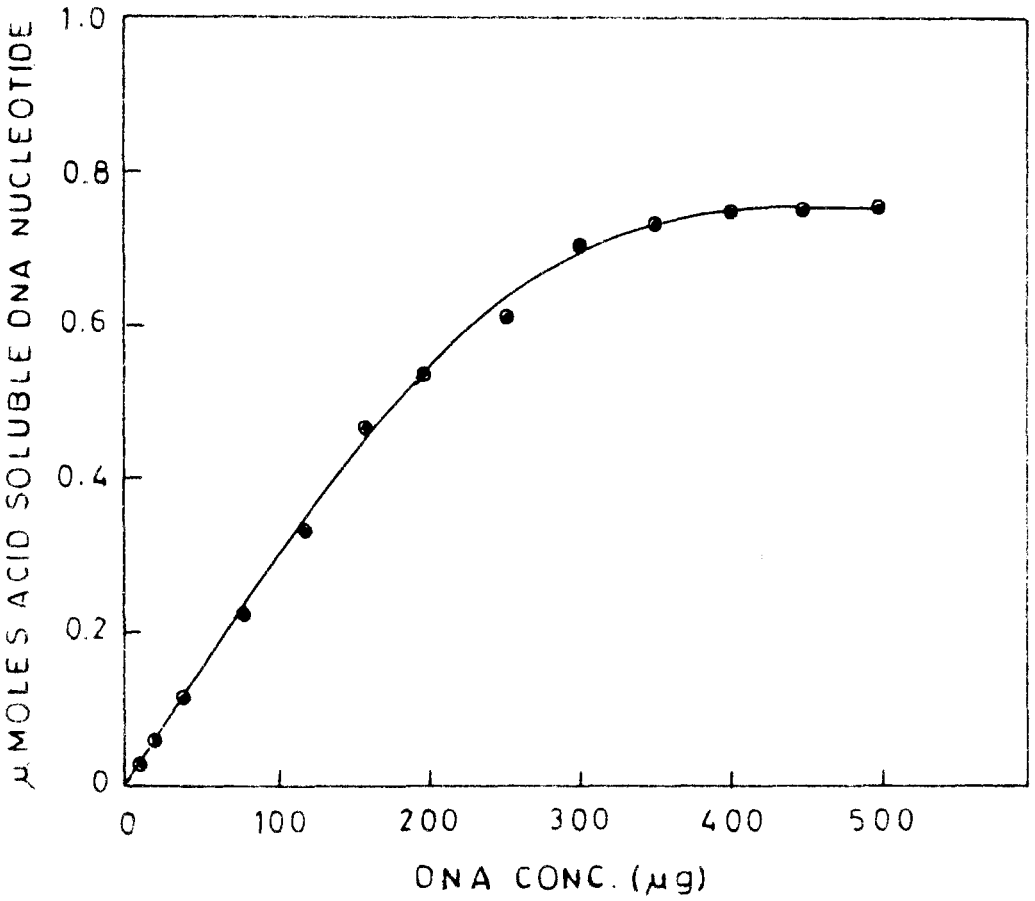
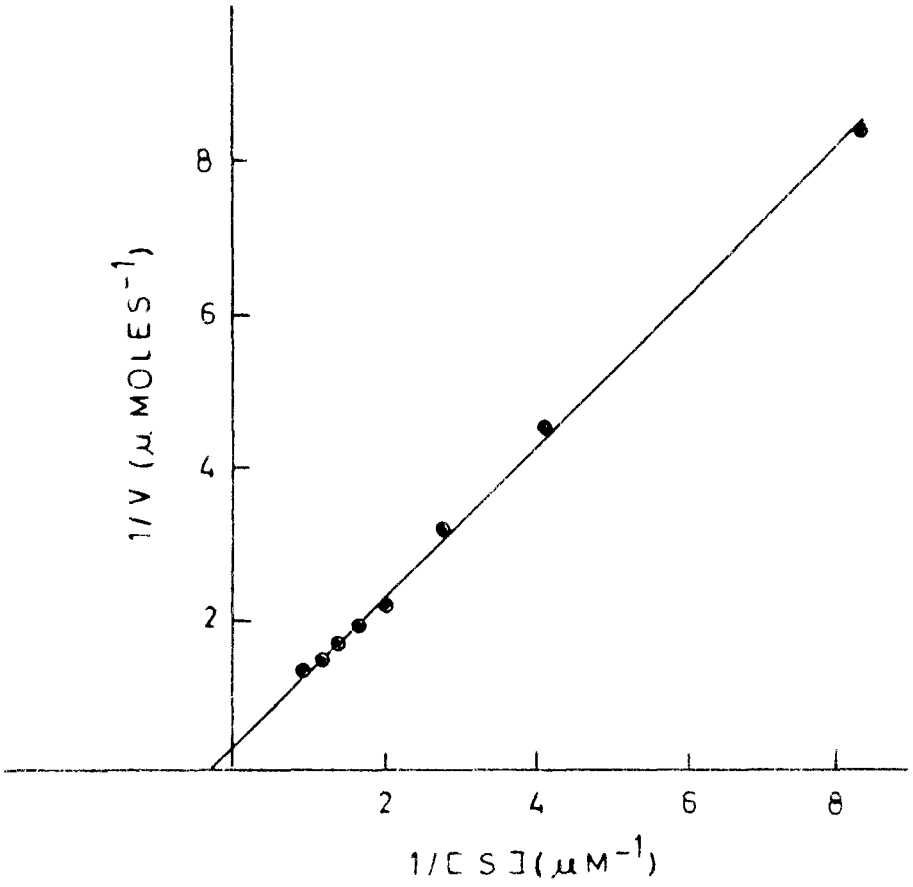

Addition of substrate
Denatured DNA
Native DNA
Addition of substrate
150
100
50
0
0
60
120
180
DNA MADE ACID SOLUBLE (%)
INCUBATION TIME (MIN)

Figure - 10. Effect of substrate concentration upon enzyme activity - Alkali denatured DNA was used. The enzyme was assayed as described for earlier experiments. Lineweaver - Burk plot was obtained by the method of least squares for the determination of affinity constant.


μ MOLES ACID SOLUBLE DNA NUCLEOTIDE
1.0
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
DNA CONC. (μg)


1/V (μ MOLES^{-1})
8
6
4
2
2
4
6
8
1/[S] (μM^{-1})

the native DNA sample presumably contains one or more single stranded fractions which were hydrolysed by the enzyme, or it contains certain denatured regions in the native molecule that are susceptible to the action of the enzyme. The former possibility was ruled out by hydroxyapatite chromatography of native DNA when no material was eluted as single stranded molecules.

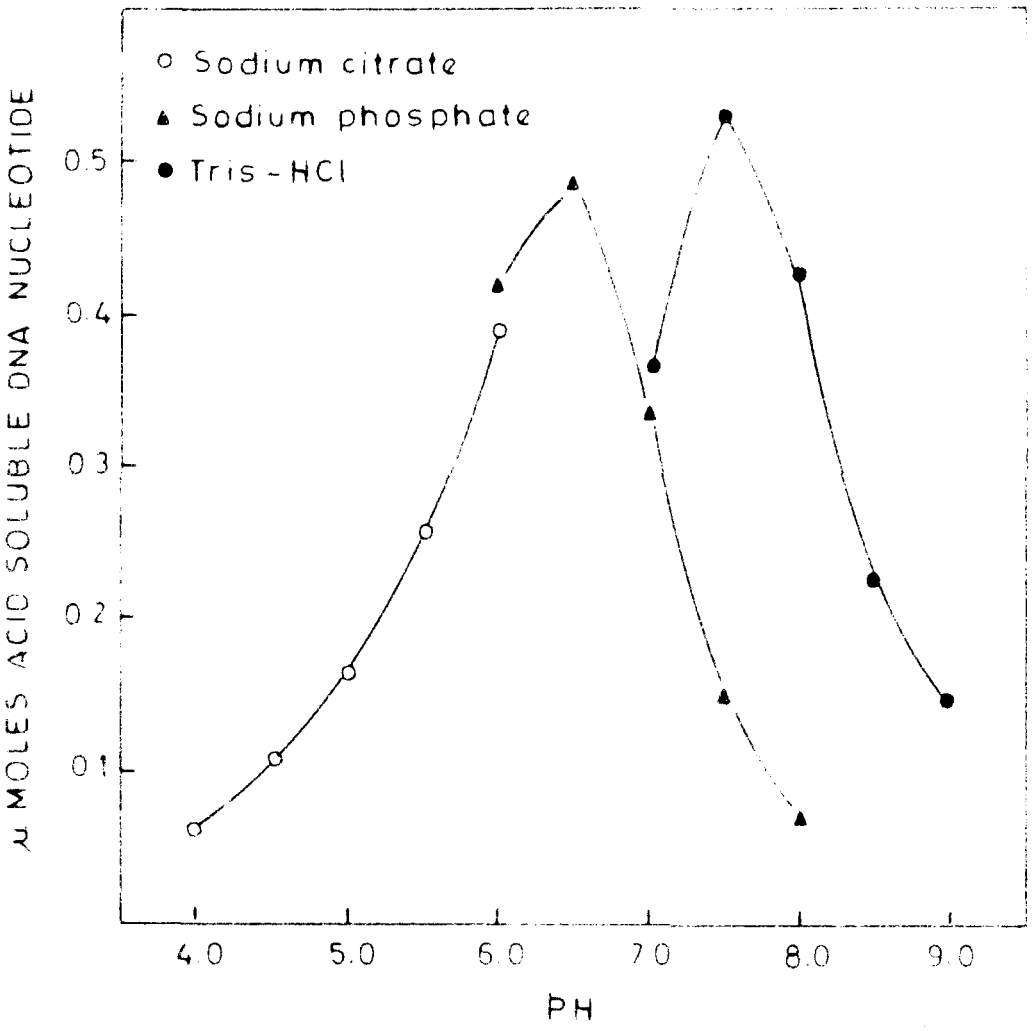
Effect of substrate concentration on enzyme activity and determination of affinity constant.

The enzyme was assayed under standard assay conditions with increasing concentrations of denatured DNA in the incubation mixture. The results are shown in Fig.10a. The apparent Km for denatured DNA was determined by the Lineweaver - Burk plot[114] of the data obtained in Fig. 10a. The straight line was interpolated by the method of least squares (Fig. 10b). The concentration of DNA giving half maximal velocity in terms of acid soluble nucleotide produced was 3.33×10^{-6}M.

Effect of pH

The rate of hydrolysis of denatured DNA in various buffers of different pH is shown in Fig. 11. The enzyme has significant activity upon denatured DNA over a broad range of pH, but is most active from pH 6.5 to 8.0. The nature of the buffering species itself is important for enzymatic activity. At pH 7.5 the enzyme

Figure - 11. pH-activity profile of pea seed nuclease - Assays were performed as described earlier. The various buffers used were sodium citrate (pH 4-6), sodium phosphate (pH 6-8) and Tris-HCl (pH 7-9). In all cases the final concentration of buffer was 0.05M. $MgCl_2$ was included in every determination at 1 mM. 0.4 unit of enzyme (fraction IV, sp. act. 62) was used. The pH refers to the value for 0.2M buffer measured at room temperature.


o Sodium citrate
▲ Sodium phosphate
● Tris - HCl
μ MOLES ACID SOLUBLE DNA NUCLEOTIDE
0.1
0.2
0.3
0.4
0.5
4.0
5.0
6.0
7.0
8.0
9.0
PH

is maximally active in tris-HCl buffer. In potassium phosphate buffer or saline sodium citrate at pH 7.5 the enzymatic activity is 30% or 60% respectively, of that in tris-HCl buffer. Somewhat similar behaviour with pH was observed by Linn and Lehman[44] for Neurospora crassa nuclease and by Holloman and Holliday[69] with Ustilago maydis single stranded specific nuclease.

Effect of temperature

Fraction IV was assayed between a temperature range of 20-80°. Maximum activity was observed at 45° with denatured DNA (Fig. 12). With native DNA the activity increased slowly showing optimal degradation at 60°.

Von-Hippel and Felsenfeld[113] and Wingert and Von-Hippel[116] have postulated that at temperatures below Tm, A, T-rich regions undergo local strand separation to a greater extent than G, C-rich regions (i.e. structural breathing). In effect the A, T-rich regions of double stranded DNA possess a certain degree of single stranded character that depends on the length of the A, T-rich region, temperature and ionic strength. By manipulating reaction conditions, it should be possible to enhance or suppress the single strand character of such regions. The continuous increase in the activity of the enzyme upto a temperature higher than with single stranded material can be attributed to the strand separation of A, T-rich regions caused at relatively elevated temperatures. For

Figure - 13. Effect of temperature upon nuclease activity - Enzyme was assayed under the standard conditions except the incubation which was carried at the temperatures indicated.

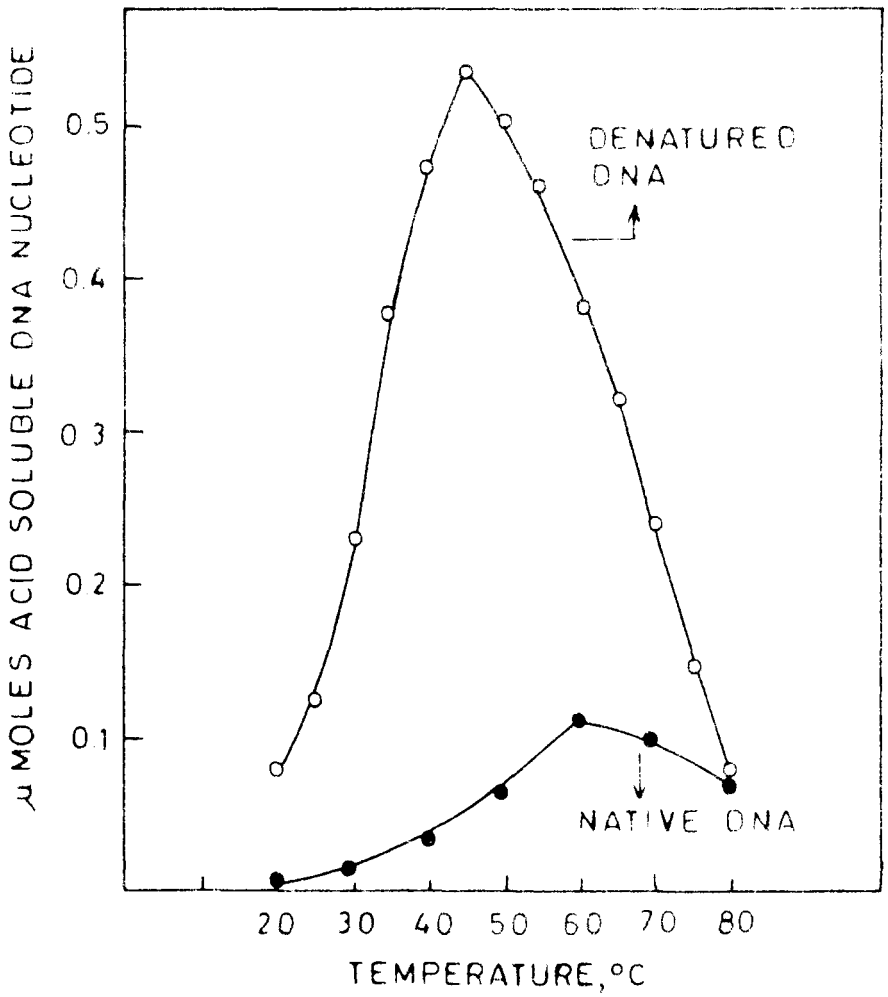
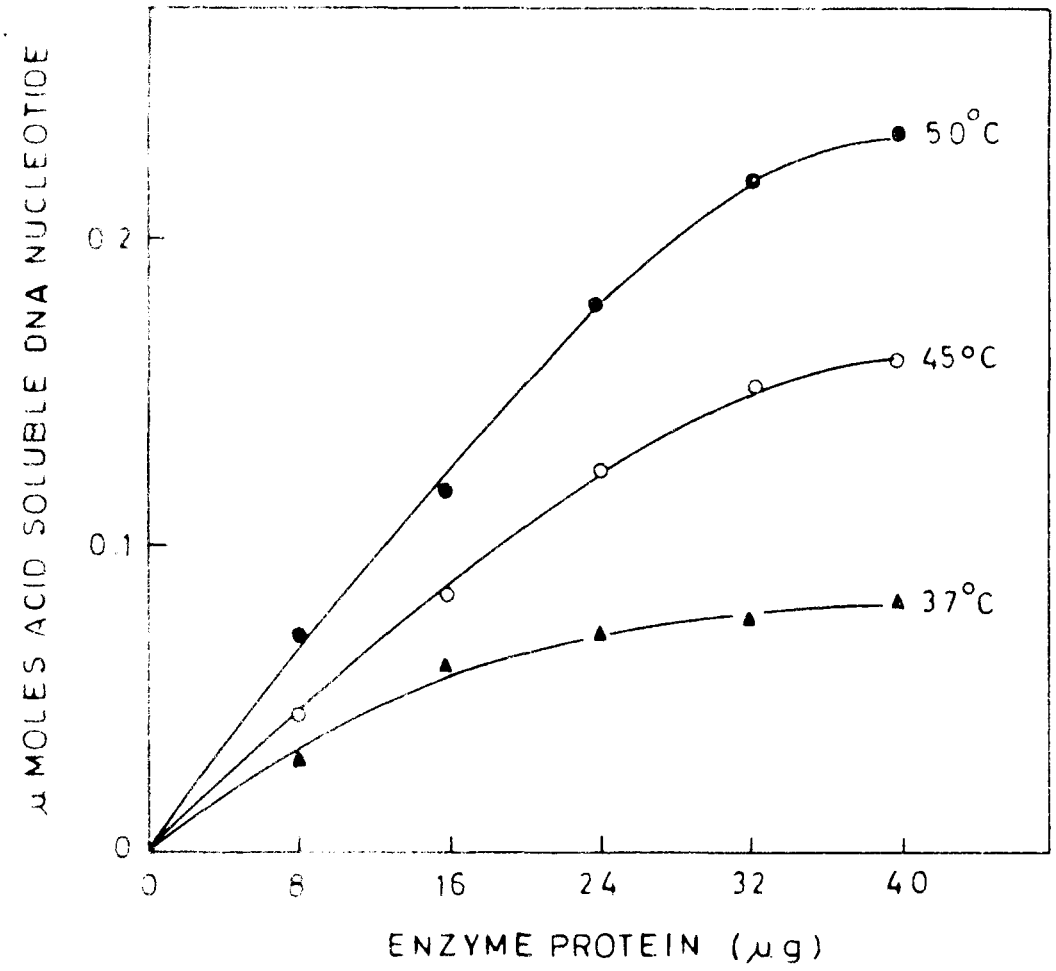

μ MOLES ACID SOLUBLE DNA NUCLEOTIDE
0.5
0.4
0.3
0.2
0.1
DENATURED DNA
NATIVE DNA
20
30
40
50
60
70
80
TEMPERATURE, °C

Figure - 13. Enzymatic hydrolysis of native DNA at elevated temperatures below Tm - Native DNA was treated with increasing concentrations of enzyme (Fraction IV, sp. act. 62) and 1 ml aliquots of reaction mixture were incubated at 37°, 45° and 50°. 20% glycerol was added to protect the enzyme from inactivation at higher temperatures. Other details were same as in standard procedure.


μ MOLES ACID SOLUBLE DNA NUCLEOTIDE
0.2
0.1
0
50°C
45°C
37°C
0
8
16
24
32
40
ENZYME PROTEIN (μg)

this reason the experiment shown in Fig. 13 was done, in which activity of the enzyme towards native DNA was determined with increasing amounts of enzyme concentration at various temperatures below Tm. It was observed that the extent and rate of hydrolysis of DNA increased as the temperature of incubation increased from 37° to 50°. The structural breathing of DNA is augmented by the increase in temperature and at 50° there is sufficient strand separation in comparison with 45° or 37°. Accordingly the pea seed nuclease being specific for single stranded DNA causes the degradation of native DNA at such regions. The results obtained in this experiment are thus consistant with the results of Fig. 12. It is therefore suggested that the degradation of native DNA by the enzyme is mainly directed towards denatured and low melting regions in the molecule and the activity of the enzyme can be a measure of the single stranded areas in a normally native structure.

Effect of salt upon the activity of enzyme

Table 3 shows the effect of increasing concentrations of sodium chloride upon denatured and native DNA degrading activity of the enzyme. The activity upon denatured DNA shows a slight increase upto 0.1M NaCl and thereafter decreases slowly with 65% being retained in presence of 0.2M NaCl. This unusual salt sensitivity could be due to a reduced affinity of enzyme towards DNA at high ionic strength. However, the activity of the enzyme towards native DNA is much more sensitive to the presence of salt

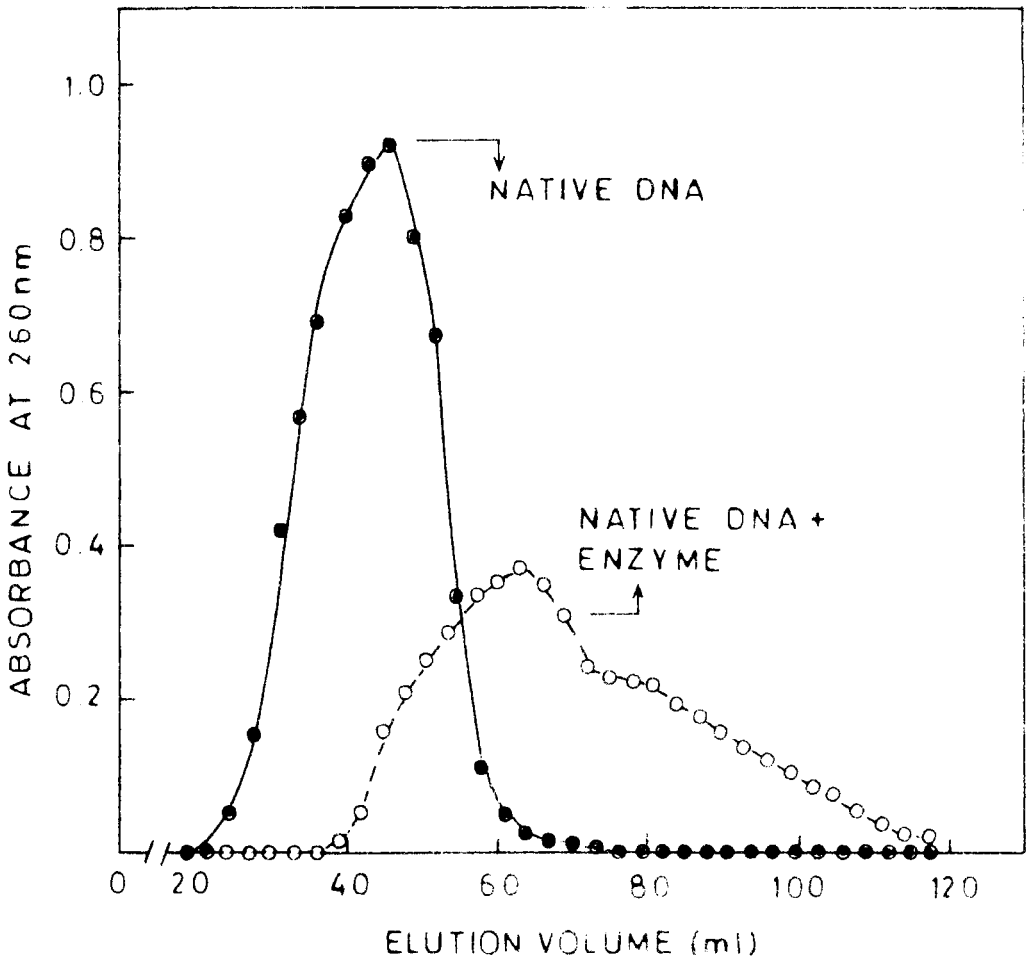
TABLE - 4

EFFECT OF INCREASING NaCl CONCENTRATION ON THE DEGRADATION OF NATIVE AND DENATURED DNA BY PEA SEED NUCLEASE.

0.6 unit/ml of enzyme (sp. act., 62) was used to assay the enzyme. The other reaction conditions are given in the text.

CONCENTRATION OF NaCl	PERCENT OF CONTROL	
	DENATURED DNA	NATIVE DNA
CONTROL (-NaCl)	100	100
0.05M	116	73
0.10M	116	63
0.15M	83	35
0.20M	65	15

Figure - 14. Chromatography of enzymatically treated native DNA on Biogel - A - The column (2 x 30 cm) was equilibrated with 0.1M Tris-HCl buffer, pH 7.0; 0.5M NaCl and 1 x 10^{-3}M EDTA. 1 mg DNA in TNE (0.01M tris-HCl, 0.1M NaCl and 2 x 10^{-4}M EDTA, pH 7.5) was applied to the column. For the enzyme treated sample the incubation mixture in 1 ml contained 1 mg in TNE, 0.05M tris-HCl, pH 7.5, 1 mM $MgCl_2$ and 3.6 units of enzyme (Fraction IV, sp. act. 62). Incubation was done at 37° for 1 hr. Before applying on the column NaCl was added to both the samples to a final concentration of 1M. 3 ml fractions were collected at a flow rate of 20 ml/hr.


1.0
0.8
0.6
0.4
0.2
ABSORBANCE AT 260nm
NATIVE DNA
NATIVE DNA +
ENZYME
0
20
40
60
80
100
120
ELUTION VOLUME (ml)

and decreases continuously till only 15% is retained at 0.2M. The plausible explanation of this decrease of native DNA degrading activity can be the salt suppression of the degree of single strandedness in the native molecule. Salt strengthens the forces stabilizing DNA in its native form and disallows any structural breathing[114,115]. As a result there are fewer denatured regions available for the nuclease to act.

Chromatography of enzymatic products of native DNA on Biogel A

Native DNA (1 mg) was chromatographed on a Biogel A-15m column (2 x 30 cm) equilibrated with 0.1M tris-HCl buffer, pH 7.5, 0.5M NaCl and 1×10^{-3}M EDTA. It appeared as a single sharp peak in the early fractions. The same preparation of DNA was then treated with excess enzyme in order to obtain a limit digest by incubating for 1 hr at 37°. The limit digest of enzymatic hydrolysis was then applied to the column. The hydrolysate appeared as a broad peak (Fig. 14) indicating that the molecular size of the DNA products was lower than the untreated DNA and varied in size considerably. Native DNA cannot be retained by Biogel A and is thus eluted in the void volume (Vo)[117]. Presumably the enzyme cleaves the native molecule to segments which are small enough to loose their rigidity and are thus able to enter the gel.

Effect of metals and other agents on the nuclease activity

The effect of divalent metal ions and various other agents is shown in table 5. Fraction IV was dialysed against EDTA and redialysed further without EDTA in order to remove any contaminating metal ion before testing with different agents. The enzyme does not demonstrate an absolute requirement for an added divalent cation. The addition of Mg^{++} and Ca^{++} results in 40% and 60% stimulation respectively. Hg^{++} was a strong inhibitor. Salt does not have any effect on the degradation of denatured DNA, whereas it inhibited the hydrolysis of native DNA. This is in agreement with the previous results (Table 4). Among the chelating agents 8-hydroxyquinoline was inhibitory whereas EDTA had no effect at the concentration tested. The observed inhibition of pea seed nuclease in the presence of 8-hydroxyquinoline but not with EDTA as well as the lack of an absolute requirement for the added divalent cation for activity suggest that the free metal is not required for enzyme function and that the inhibition by 8-hydroxyquinoline is probably through direct interaction with the enzyme possibly with a bound metal. Sulfhydral reagents like N-ethylmaleimide (NEM), Dithiobis-2-nitrobenzoic acid (DTNB) and parachloromercuribenzoate (PCMB) had no effect. Mercaptoethanol and other -SH donors were also without effect. Thus the enzyme does not require an -SH group for its activity. Addition of yeast RNA to the reaction mixtures did not effect the extent of degradation

TABLE - 5

EFFECT OF DIFFERENT AGENTS ON THE ACTIVITY OF PEA SEED NUCLEASE ON DENATURED AND NATIVE DNA.

Assays were done in the presence of various agents. Incubations were for 1 hr. at 37°. Fraction IV was dialysed against 0.02M tris-HCl, pH 7.5 containing 1×10^{-4}M EDTA and redialysed against buffer only before use in the assay. 0.3 unit/ml of enzyme (sp. act., 62) was added.

AGENT	FINAL CONC	PERCENT OF CONTROL	
		DENATURED DNA	NATIVE DNA
Control	-	100	100
$MgCl_2$	5×10^{-3}M	141	149
$MnCl_2$	5×10^{-3}M	100	115
$CoCl_2$	5×10^{-3}M	72	101
$HgCl_2$	5×10^{-3}M	0.0	0.0
$CaCl_2$	5×10^{-3}M	159	134
KCl	0.15M	141	66
NaCl	0.15M	108	40
EDTA	1×10^{-3}M	118	100
8-hydroxyquinoline	1×10^{-3}M	50	70
Sod. Pot. tartarate	5×10^{-2}M	106	-
Sod. dihydrogen phosphate	5×10^{-2}M	13	-
Iodoacetate	5×10^{-3}M	96	-
Mercaptoethanol	1×10^{-3}M	91	-
Glutathione	1×10^{-3}M	94	-
Cysteine hydrochloride	1×10^{-3}M	104	-
N-ethylmaleimide	1×10^{-3}M	94	-
DTNB	1×10^{-3}M	82	-
PCMB	1×10^{-3}M	86	-
8-Azaguanine	1×10^{-3}M	100	-
Yeast RNA	200 ug/ml	100	-

TABLE - 6

EFFECT OF INCREASING CONCENTRATION OF Ca^{++} AND Mg^{++} ON THE NUCLEASE ACTIVITY.

0.6 unit/ml of enzyme was used. The other conditions of assay were as in Table - 5.

FINAL CONCENTRATION (M)	PERCENT OF CONTROL	
	Mg^{++}	Ca^{++}
Control	100	100
5×10^{-6}	110	100
1×10^{-5}	116	110
5×10^{-5}	123	116
1×10^{-4}	136	126
5×10^{-4}	140	174
1×10^{-3}	138	182
5×10^{-3}	119	145
1×10^{-2}	100	133
5×10^{-2}	21	-
1×10^{-1}	1	-

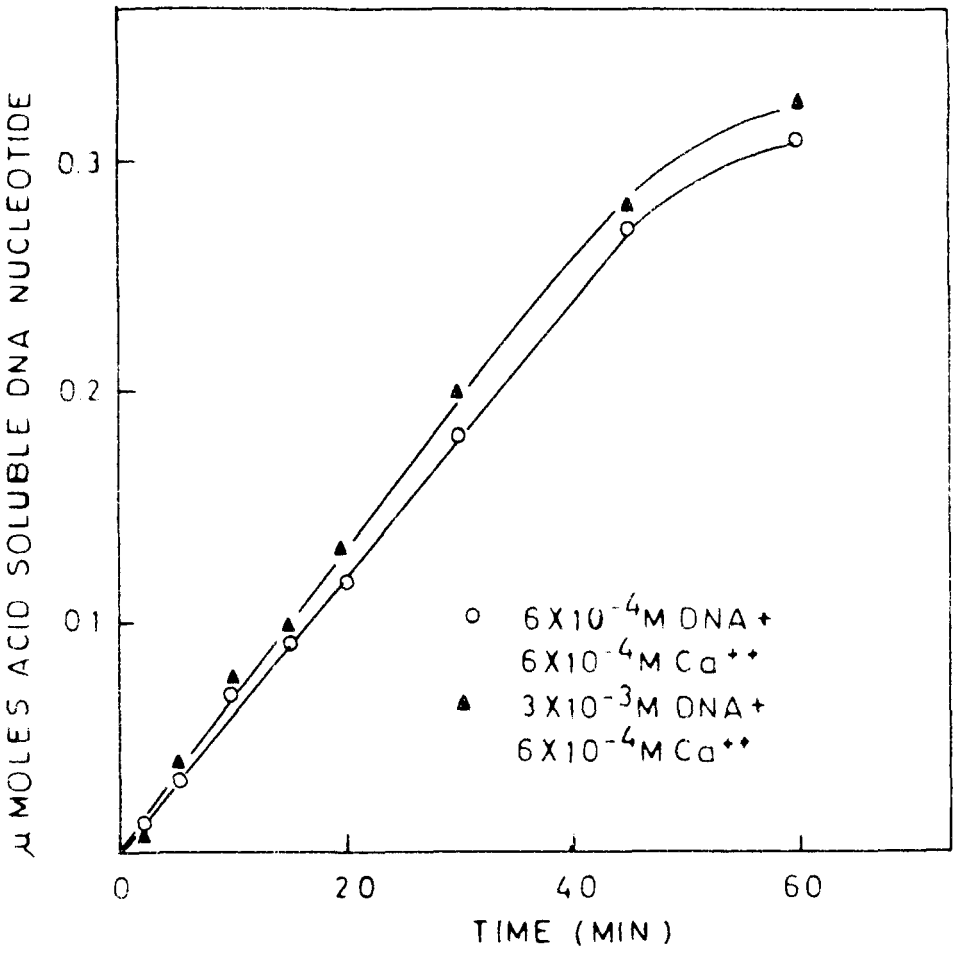
TABLE - 7

REVERSAL OF METAL ACTIVATION OF THE ENZYME BY EDTA

0.6 unit/ml enzyme was used. The other conditions were the same as in Table - 5.

TREATMENT	PERCENT OF CONTROL
Control	100
Mg^{++}, 5×10^{-4}M	140
Ca^{++}, 5×10^{-4}M	170
EDTA, 1×10^{-3}M	100
Mg^{++}, 5×10^{-4}M Ca^{++}, 5×10^{-4}M	161
Mg^{++}, 5×10^{-4}M EDTA, 1×10^{-3}M	92
Ca^{++}, 5×10^{-4}M EDTA, 1×10^{-3}M	96
Mg^{++}, 5×10^{-4}M Ca^{++}, 5×10^{-4}M EDTA, 1×10^{-3}M	81

Figure - 15. Effect of Ca^{++} upon the rate of degradation of denatured DNA - The incubation mixture contained $CaCl_2$ and DNA in molar ratios of 1:1 (O—O) and 1:5 (Δ—Δ). 0.6 unit of enzyme was used. The other ingredients being same as for the standard assay mixture. Ca^{++} was present at a concentration of 6×10^{-4} M. Aliquots of 1 ml from the incubation mixture which was incubated at 37° were removed at indicated time interval and treated in the usual manner.


μ MOLES ACID SOLUBLE DNA NUCLEOTIDE
0.3
0.2
0.1
0
20
40
60
TIME (MIN)
6X10⁻⁴M DNA + 6X10⁻⁴M Ca⁺⁺
3X10⁻³M DNA + 6X10⁻⁴M Ca⁺⁺

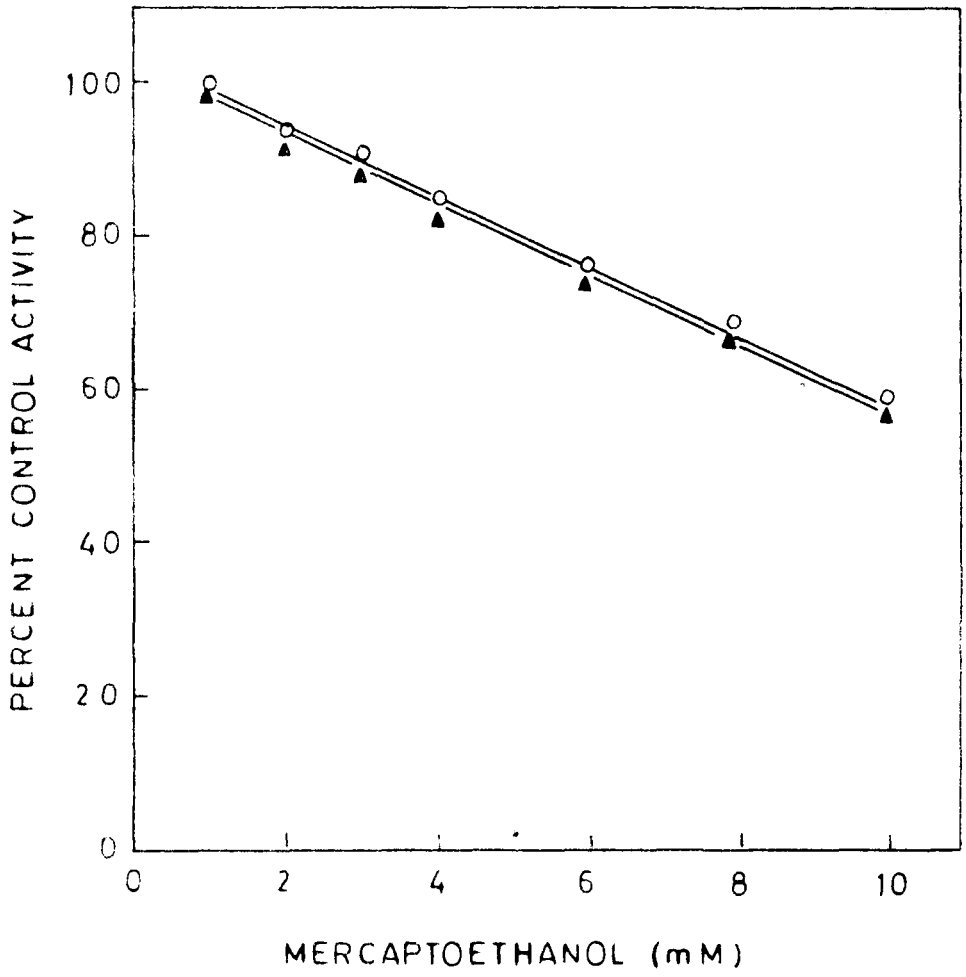
of DNA. Thus RNA does not bind to the enzyme. In another experiment (results not shown) several monoribonucleotides were tested and were found to be without effect on the enzyme activity. This is in contrast with the behaviour of plant nuclease I from tobacco cell cultures[49].

In Table 6 is shown the effect of increasing concentrations of Ca^{++} and Mg^{++} ions upon nuclease activity. The optimum requirement was found to be 1×10^{-3}M and 5×10^{-4}M respectively for Ca^{++} and Mg^{++}. When Mg^{++} and Ca^{++} were added to the reaction mixture simultaneously no synergistic effect was seen (Table 7). When EDTA was added with Ca^{++} or Mg^{++} alone or in the presence of both, the stimulatory effect of the metals was reversed.

In order to determine whether the activation of nuclease activity by metal ions is due to the binding of the cation to the enzyme or DNA the experiment shown in Fig.15 was performed. In one reaction mixture Ca^{++} was present in stiochiometric amounts to the substrate. In another the concentration of the substrate was increased five fold and the rate of degradation of DNA followed as a function of time. In both cases the rate of degradation of DNA remained same upto 60 minutes. The results suggest that stimulation of the enzyme activity by Ca^{++} is due to its interaction with the enzyme rather than with DNA.

The fact that the enzymatic degradation of native as well as denatured DNA does not require a metal ion and is not inhibited

Figure - 16. <u>Effect of mercaptoethanol upon the nuclease activity</u> - Enzyme was assayed with the increasing concentrations of 2-mercaptoethanol 0.4 and 0.6 unit of enzyme were used for denatured DNA (○——○) and native DNA (▲——▲) respectively.


PERCENT CONTROL ACTIVITY
100
80
60
40
20
0
0
2
4
6
8
10
MERCAPTOETHANOL (mM)

by EDTA suggests that these activities are due to a single protein. Further, the stimulation by Ca^{++} and Mg^{++} and inhibition by 8-hydroxyquinoline strengthens this possibility.

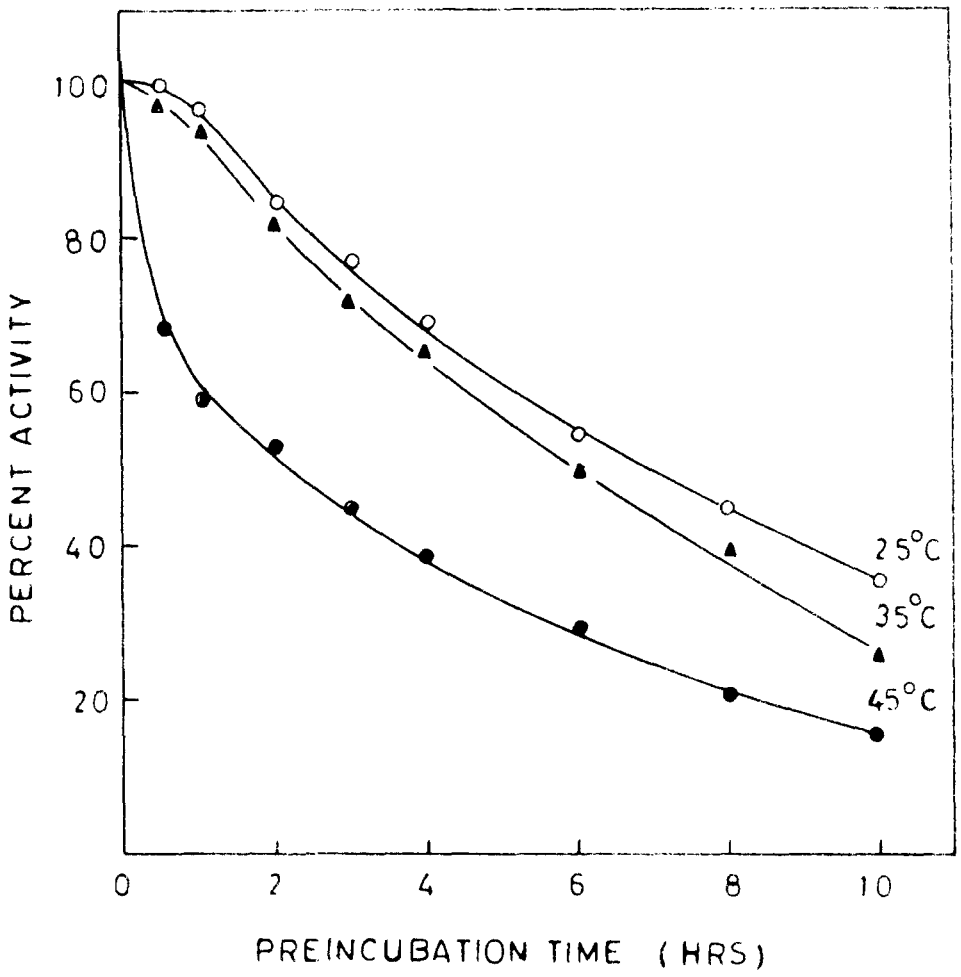
Effect of 2-mercaptoethanol on hydrolysis of native and denatured DNA

Neurospora crassa nuclease that degrades single stranded as well as native DNA has been found to be inhibited by 2-mercapto-ethanol[44]. The hydrolysis of native DNA was considerably more sensitive to the action of thiols. The pea seed nuclease was also found to be sensitive to 2-mercaptoethanol although to a lesser extent. As shown in Fig.16, 60% activity was retained in the presence of 10mM 2-mercaptoethanol. Furthermore, the extent of inhibition by the thiol reagent is similar with both native and denatured DNA. The results further strengthen the possibility that the activity of fraction IV on native and denatured DNA is due to a single enzyme.

Stability of the enzyme

The results shown in Fig. 12 and 13 indicated that the enzyme was fairly heat stable. Fig. 17 shows the effect of time of incubation of the enzyme at different temperatures upon its activity. Fraction IV was preincubated at the temperatures indicated and aliquots were removed at indicated intervals and assayed. The

Figure - 17. Temperature stability of pea seed nuclease - Fraction IV was preincubated at 25°, 35° and 45°. At indicated times 0.05 ml (0.6 unit) of enzyme were taken and assayed under the standard conditions.

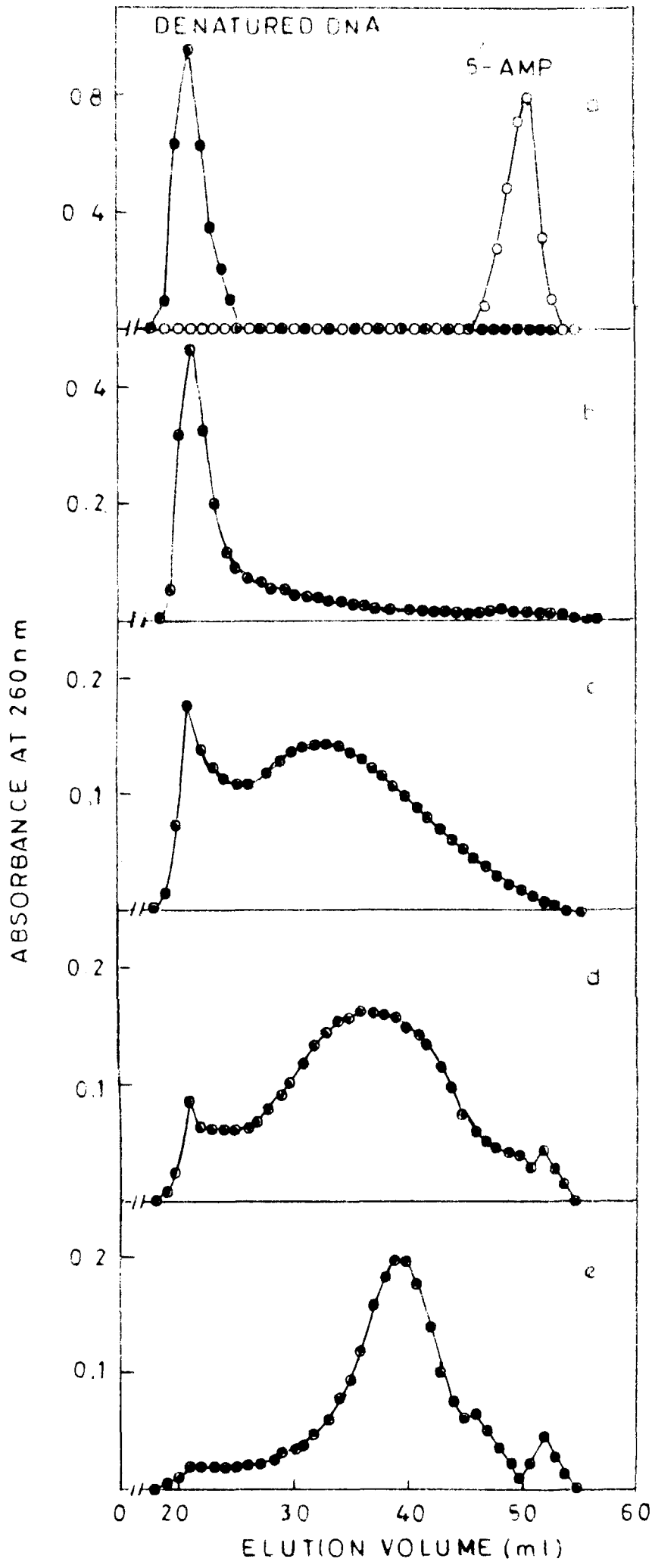

100
80
60
40
20
PERCENT ACTIVITY
0
2
4
6
8
10
PREINCUBATION TIME (HRS)
25°C
35°C
45°C

activity decreased slowly at 25° and 35° while there was a rapid decrease of activity at 45°. 50% of the enzyme activity is lost in 2 hours at 45° while it takes 7 hours for 50% loss of activity at 25° and 35°. The enzyme remains stable on storage at 4° with only little loss of activity. However, no loss of enzyme activity was observed upto 3 months when it was stored frozen in presence of 10% glycerol in 0.02M tris-HCl buffer, pH 7.5 and 1×10^{-3}M 2-mercaptoethanol.

Mode of degradation

In order to determine whether the activity of pea seed nuclease was of exonucleolytic or endonucleolytic type, calf thymus DNA was digested to different degrees of acid solubilization in standard reactions and chromatographed on a sephadex G-100 column as described by Birnboim[118]. Calf thymus DNA was denatured and was incubated with the enzyme as described in the methods. The time of incubation and the concentration of enzyme was adjusted to produce partial digests of the DNA in order to examine intermediate stages in the degradation. At intervals during the reaction 1 ml aliquots were removed and heated at 100° for 5 minutes to inactivate the enzyme. Each sample was cooled and chromatographed on a sephadex G-100 column (1.7 x 20 cm) equilibrated with 0.1M tris-HCl buffer pH 7.5. The absorbance at 260 nm of the effluent was monitored using 1 cm cells in Beckman DU-model spectrophotometer.

Figure - 18. <u>Chromatography on sephadex G-100 of denatured DNA at stages of its digestion by the nuclease</u> - The incubation mixture contained 200 ug/ml denatured DNA, 0.05M Tris-HCl buffer, pH 7.5, 1 mM $MgCl_2$ and 1.2 unit/ml of enzyme. The elution pattern shown are for (a) denatured DNA and 5'-AMP; and 1 ml aliquots of incubation mixture which was incubated at 37° for (b) 2 min. (c) 5 min. (d) 10 min. and (e) 30 min. See text for details.


DENATURED DNA
5-AMP
a
b
c
d
e
0.8
0.4
0.4
0.2
0.2
0.1
0.2
0.1
0.2
0.1
ABSORBANCE AT 260nm
0
20
30
40
50
60
ELUTION VOLUME (ml)

Denatured DNA and the mononucleotide 5'-AMP were separately chromatographed and two discrete peaks could be identified (Fig.18a). The first peak in the void volume corresponded to the position of DNA and the second peak to the mononucleotide.

When 10% of the DNA was made acid soluble during 2 minute incubation with enzyme the size of the DNA peak diminished and only slightly low molecular weight material started appearing (Fig. 18b). When 40% of the DNA was acid solubilized in 5 minutes the DNA peak had reduced considerably and a broad peak corresponding to small molecular weight fragment of DNA appeared (Fig. 18c). There was further increase in this peak and slight shift as 75% of DNA was made acid soluble in 10 minutes incubation (Fig. 18d). However, when about 95% of DNA was degraded to acid soluble material in 30 minutes incubation pronounced shift of the peak was observed, while the peak corresponding to DNA had almost vanished (Fig.18e). Only a small amount appeared as mononucleotides after 30 minutes incubation with the enzyme.

The fact that the DNA was reduced in size and the products of incubation with the enzyme, when chromatographed, eluted from the column as a single peak whose position shifted from that of the undigested DNA towards the position of mononucleotides was consistant with an endonucleolytic mode of action and the profile obtained is typical of an endonuclease.

Determination of molecular weight of pea seed nuclease by gel filtration

The molecular weight of pea seed nuclease was determined by gel filtration according to the method of Andrews[119] using a sephadex G-200 (2 x 52 cm) column. The column was equilibrated with 0.02M tris-HCl buffer pH 7.5, 0.1M NaCl. 3 mg of fraction III (DEAE-cellulose fraction) was chromatographed on the column previously calibrated with marker proteins, namely, Cytochrome-C, -Chymotrypsinogen - A, Ovalbumin and Bovine Serum Albumin, of known molecular weight and Stoke's radius. Elution was performed with the same buffer and fractions of 2 ml each were collected at a flow rate of 15 ml/hr. The protein in the fractions was determined by Lowry's method and enzyme activity was determined by usual standard assay procedure. Blue Dextran 2000 (Pharmacia) was measured at 625 nm. Elution profile of Blue Dextran, marker proteins and pea seed nuclease is shown in Fig. 19a and 19b. The gel filtration data of pea seed nuclease and marker proteins in terms of Kd, Kav and Ve/Vo are presented in Table 8. The elution volumes were reproducible. A plot of Ve/Vo versus logarithm of molecular weight (Fig. 20) according to the procedure of Andrews[119] gave a straight line. Blue Dextran was used to find the void volume (Vo) whereas the total volume (Vt) of the column was determined directly with water. The elution volume Ve of a given solute zone was taken in all cases as the effluent peak position

Figure-19(a). Gel filtration behaviour of protein standards on sephadex G-200 column - About 5-10 mg of proteins were chromatographed on the column (2 x 52 cm) in 0.02M Tris-HCl buffer, 0.1M NaCl and eluted in 2 ml fractions at a flow rate of 15 ml/hr.

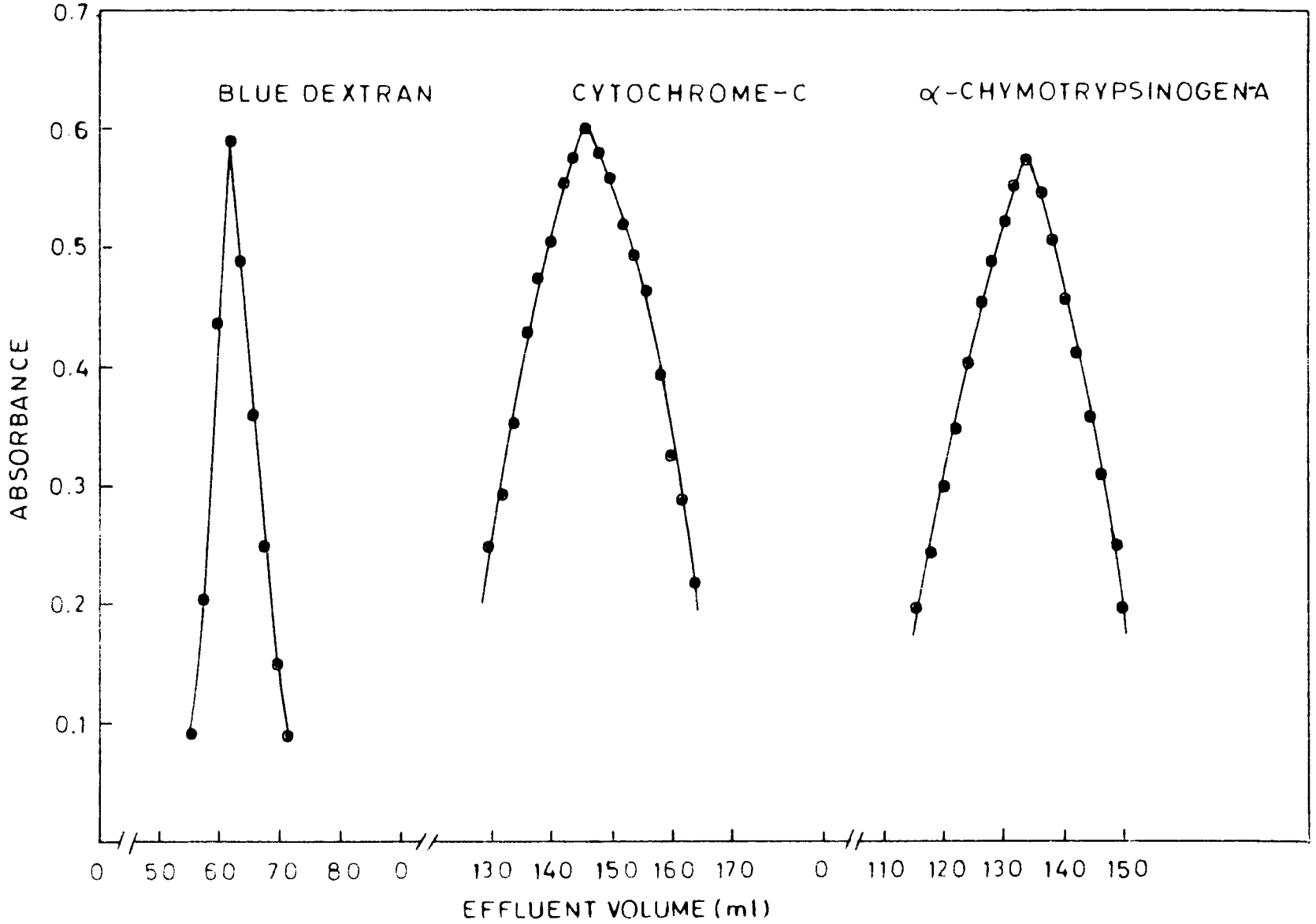

BLUE DEXTRAN
CYTOCHROME-C
α-CHYMOTRYPSINOGEN-A
ABSORBANCE
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
50
60
70
80
0
130
140
150
160
170
0
110
120
130
140
150
EFFLUENT VOLUME (ml)

Figure-19(b). Gel filtration behaviour of protein standards and pea seed nuclease on sephadex G-200 - The experimental conditions were same as described for Fig.19(a). Enzyme activity in the different fractions was assayed as described in "methods".

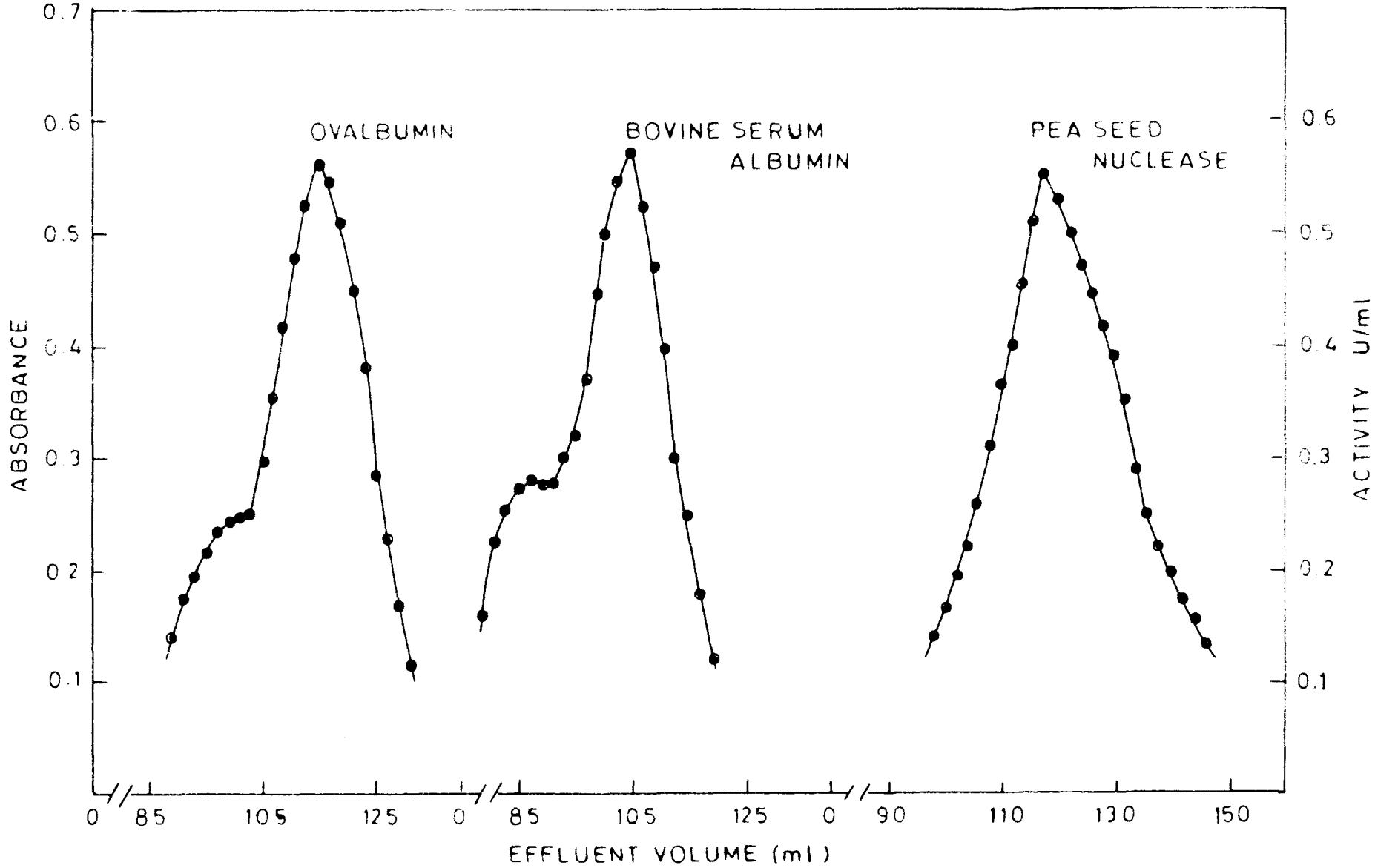

OVALBUMIN
BOVINE SERUM ALBUMIN
PEA SEED NUCLEASE
ABSORBANCE
ACTIVITY U/ml
EFFLUENT VOLUME (ml)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
85
105
125
90
110
130
150

Figure - 20. Estimation of molecular weight of pea seed nuclease by gel filtration on sephadex G-200 - The elution data of the table 8 were treated according to the method of Andrews[119]. Ve/Vo, Vs, log M was plotted by the method of least squares.

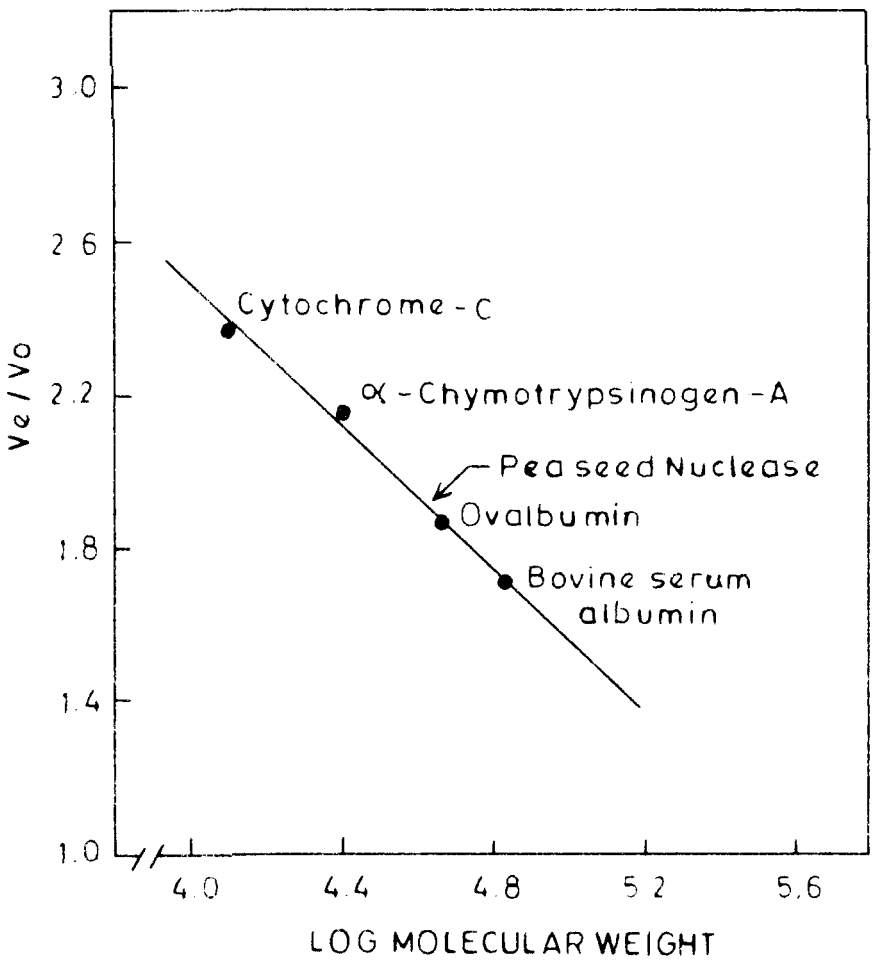

3.0
2.6
2.2
1.8
1.4
1.0
Ve / Vo
Cytochrome - C
α - Chymotrypsinogen - A
Pea seed Nuclease
Ovalbumin
Bovine serum albumin
4.0
4.4
4.8
5.2
5.6
LOG MOLECULAR WEIGHT

Figure - 21. Estimation of Stoke's radius of pea seed nuclease - The gel filtration data of table 8 were treated according to the correlation of Porath[120]. The data was plotted by the method of least squares.

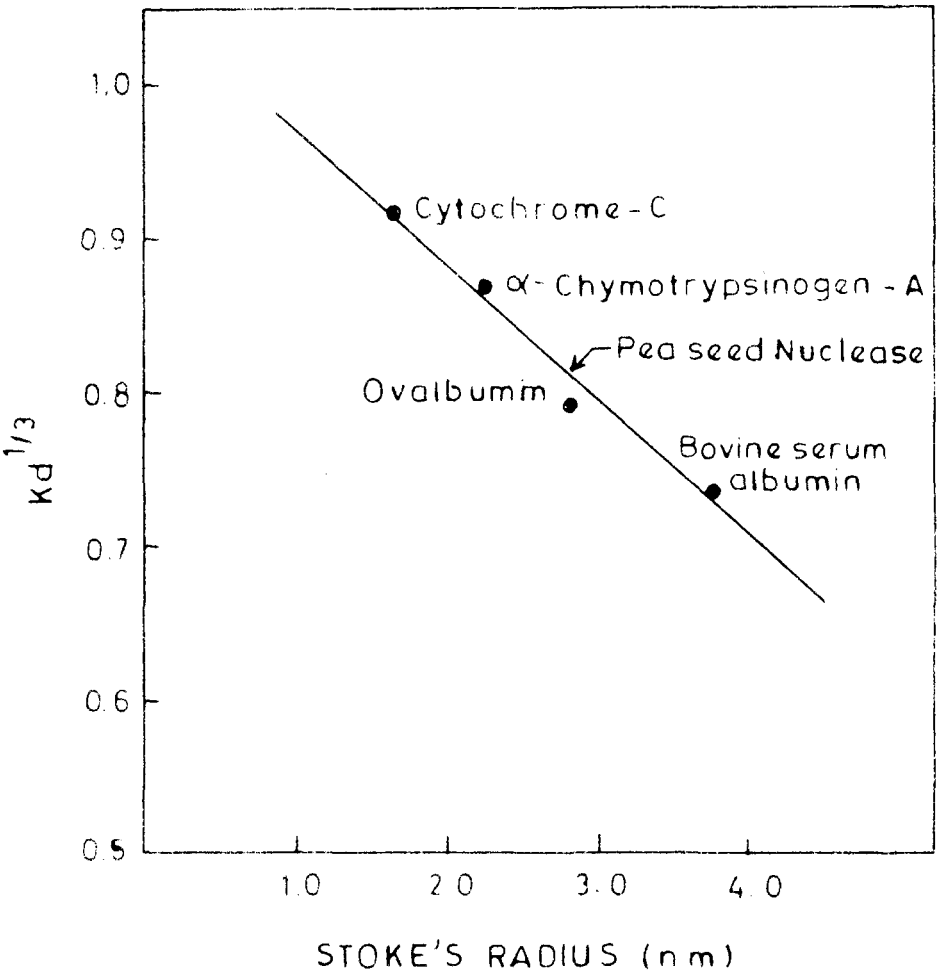

1.0
0.9
0.8
0.7
0.6
0.5
$Kd^{1/3}$
Cytochrome-C
α-Chymotrypsinogen-A
Pea seed Nuclease
Ovalbumin
Bovine serum albumin
1.0
2.0
3.0
4.0
STOKE'S RADIUS (nm)

TABLE - 8

GEL FILTRATION DATA OF PEA SEED NUCLEASE AND STRANDED PROTEINS

Proteins	Mol.wt.	Ve/Vo	Stoke's Radii(nm)	Kd	$(Kd)^{1/3}$
Cytochrome-C	12,400	2.355	1.64	0.777	0.9193
-Chymotrypsinogen-A	25,741	2.161	2.24	0.666	0.8733
Ovalbumin	46,000	1.855	2.73	0.490	0.7889
Bovine Serum Albumin	69,000	1.693	3.55	0.398	0.7356
Pea seed nuclease	42,100	1.903	2.91	0.518	0.8031

of the solute. The void volume was occasionally checked during the column chromatography of the marker proteins. There was no detectable change in Vo and Vt. The parameters Kd and Kav were calculated as defined by following equations[120,121].

$$Kd = (Ve - Vo)/Vi$$

$$Kav = (Ve - Vo)/(Vt - Vo)$$

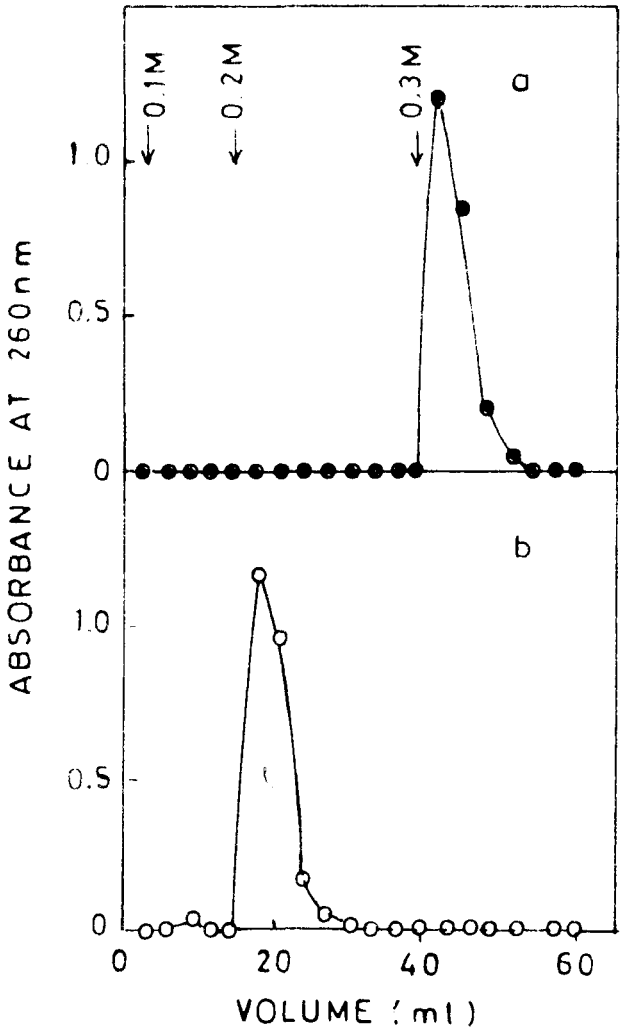
Vt, Vo and Ve have been described above. Kd is the distribution coefficient, Kav is the available distribution coefficient, and Vi is the inner volume which was determined by using tyrosine.

The gel filtration data were analysed using procedures including those of Porath[120], Laurent and Killander[121] and Ackers[122] by least squares method. The molecular weight of the enzyme was calculated to be 42,000 by Andrews procedure[119]. The Stoke's radius of pea seed nuclease was determined to be 2.80 nm (Fig. 21) as calculated by the method of Porath[120]. This value agreed with the radii calculated by the procedure of Laurent and Killander[121] and Ackers[122]. The average value comes out to be 2.819 nm (Table 8).

<u>Secondary structure of dimethyl sulfate treated and cross linked DNA and its enzymatic degradation</u>

Experiment with crude extract had earlier shown that DNA

Figure - 22. Chromatography of native and denatured DNA on hydroxyapatite - The column (1 x 3 cm) was equilibrated with 0.01M phosphate buffer, pH 7.0 containing 1% formaldehyde. 600 ug DNA samples were applied on the column. The elution was performed with a stepwise gradient of phosphate buffer containing 1% formaldehyde and 3 ml fractions were collected. The elution pattern is shown for (a) native DNA and (b) heat denatured DNA.


0.1M
0.2M
0.3M
a
b
1.0
0.5
0
1.0
0.5
0
0
20
40
60
ABSORBANCE AT 260nm
VOLUME (ml)

alkylated with dimethyl sulfate was degraded preferentially to native DNA (Fig. 2). The partially purified enzyme also demonstrated similar behaviour (Table 10). Thus alkylation transforms the DNA into an effective substrate for the pea seed nuclease. Therefore in order to determine the changes that may have been brought about in the secondary structure of DNA on alkylation hydroxyapatite chromatography was utilized. DNA was treated with dimethyl sulfate under various conditions and subjected to hydroxyapatite chromatography in order to separate single and double stranded molecules.

In Fig. 22 is shown the elution profile of single and double stranded DNA from hydroxyapatite column where they are eluted at different molarities of phosphate buffer. Fig. 23a,b,c,d show the hydroxyapatite chromatography of DNAs alkylated with increasing molar ratios of dimethyl sulfate in the presence of 0.1M NaCl. Increasing number of DNA molecules show strand separation with concomitant decrease in the double stranded material. While only 10% molecules are denatured when DNA was alkylated with DNA nucleotide to DMS molar ratio 1:1; 46% molecules show strand separation with 1:8 molar ratio. As to the reason why all molecules are not denatured at the same time one possibility could be the heterogenous population of molecules in the DNA used in the experiment. The hypochromicity of native DNA as determined by heat denaturation was 24%, whereas for DNA alkylated with DMS

Figure-23. Chromatography of alkylated DNA on hydroxyapatite - The procedure was same as in Fig. 22. The elution pattern is shown for DNAs alkylated with DMS in molar ratios of (a) 1:1, (b) 1:2, (c) 1:4 and (d) 1:8. See text for the details of alkylation procedure.

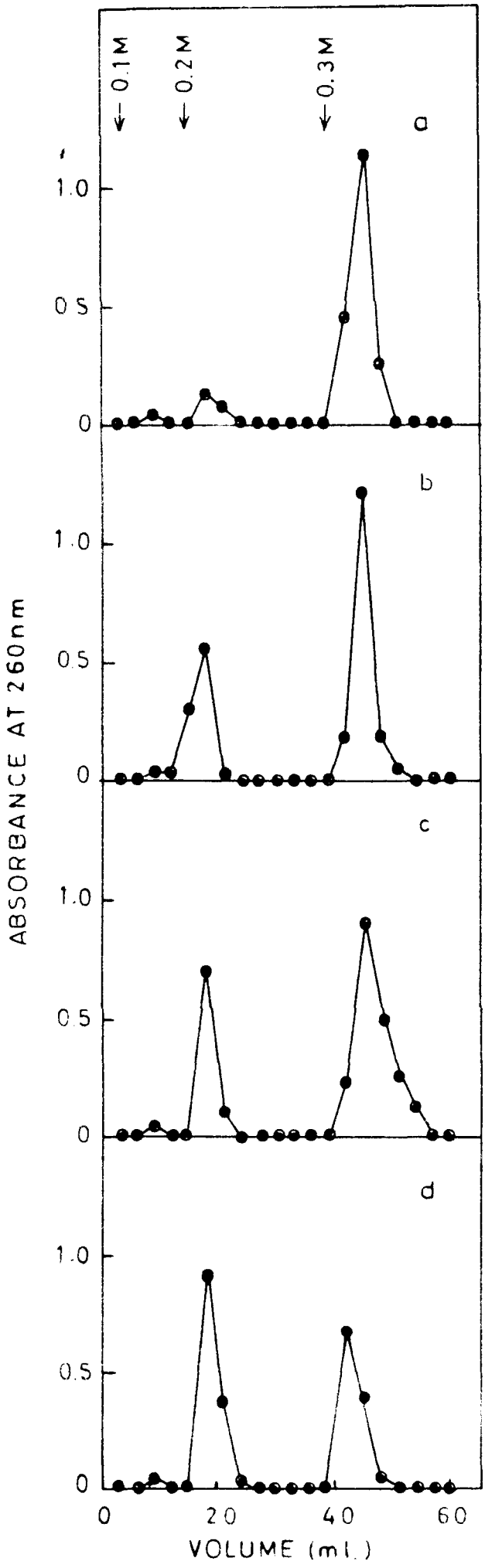
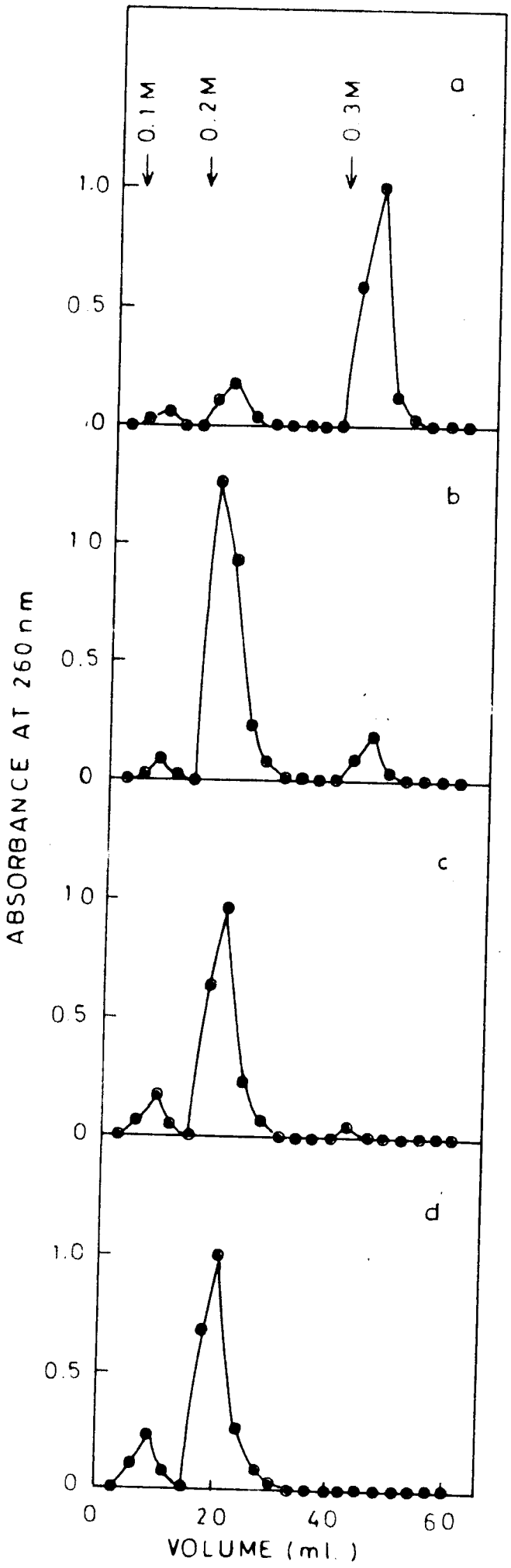

0.1M
0.2M
0.3M
a
b
c
d
1.0
0.5
0
ABSORBANCE AT 260nm
0
20
40
60
VOLUME (ml.)

Figure - 24. Chromatography of depurinated DNA on Hydroxyapatite - Depurinated DNA was obtained from alkylated samples as described in the text. The procedure was same as in Fig. 22. The elution pattern is shown for DNAs obtained on depurination of alkylated samples with DMS in molar ratios (a) 1:1, (b) 1:2, (c) 1:4, and (d) 1:8.


0.1M
0.2M
0.3M
a
b
c
d
1.0
0.5
0
ABSORBANCE AT 260 nm
0
20
40
60
VOLUME (ml.)

molar ratio of 1:1, 1:2 and 1:4 it was 23, 15, and 10.5% respectively. This observation alongwith the results of figure 23a suggests that light alkylation of DNA (e.g. DNA/DMS molar ratio 1:1) does not result in any significant denaturation. Alkaline hydrolysis of DNA alkylated with DMS upto a molar ratio of 1:4 showed negligible production of acid soluble nucleotides (Table 9). This suggested that no significant depurination of alkylated sites had occurred[123]. However, when depurinated DNAs obtained from these alkylated DNAs were subjected to alkaline hydrolysis appreciable production of acid soluble nucleotides was observed, indicating that alkylation of DNA by DMS had taken place (Table 9). This is further suggested by a control experiment where native DNA was heated at 50° for 4 hr and then treated with alkali. No production of acid soluble material was seen. In contrast when DNA was alkylated with DMS molar ratio of 1:8 (Fig. 23d) appreciable alkaline hydrolysis of alkylated as well as depurinated DNA occurred.

Hydroxyapatite chromatography of depurinated DNAs (Fig. 24) prepared by mildly heating the alkylated DNAs showed that alkylated DNA was further denatured when subjected to depurination. With a DNA nucleotide to DMS molar ratio of 1:2 only 15% of the molecules (Fig.23b) are denatured on alkylation. On depurination of the same DNA the percent denaturation reaches 90 (Fig. 24b). Subsequent higher ratios of DMS cause 100% denaturation of depurinated DNA (Fig. 24c,d).

Figure - 25. <u>Chromatography on hydroxyapatite of DNA alkylated for various periods in the presence of 0.1M and 0.01M NaCl</u> - The chromatographic procedure was same as for Fig. 22. Elution pattern is shown for DNAs alkylated with DMS in a DNA nucleotide/DMS molar ratio of 1:4 for (a) 2 hr. in 0.01M NaCl, (b) 5 hr. in 0.01M NaCl, (c) 10 hr. in 0.01M NaCl and (d) 10 hr. in 0.1M NaCl.

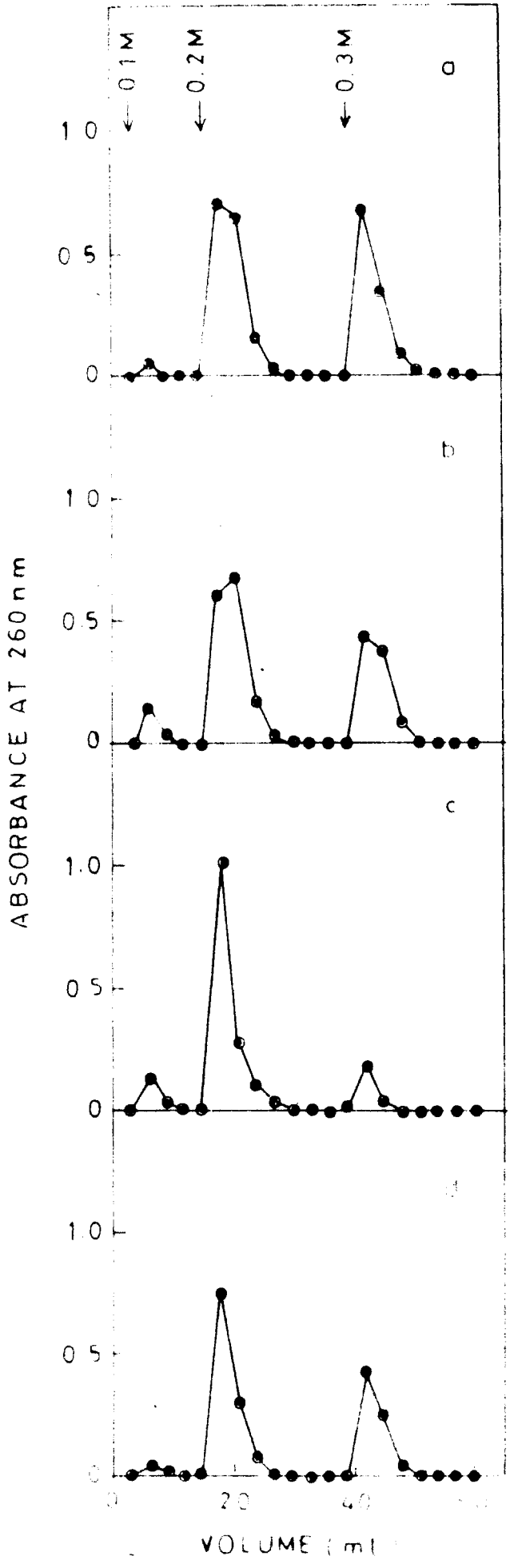

0.1M
0.2M
0.3M
a
b
c
1.0
0.5
0
ABSORBANCE AT 260nm
20
40
VOLUME (ml)

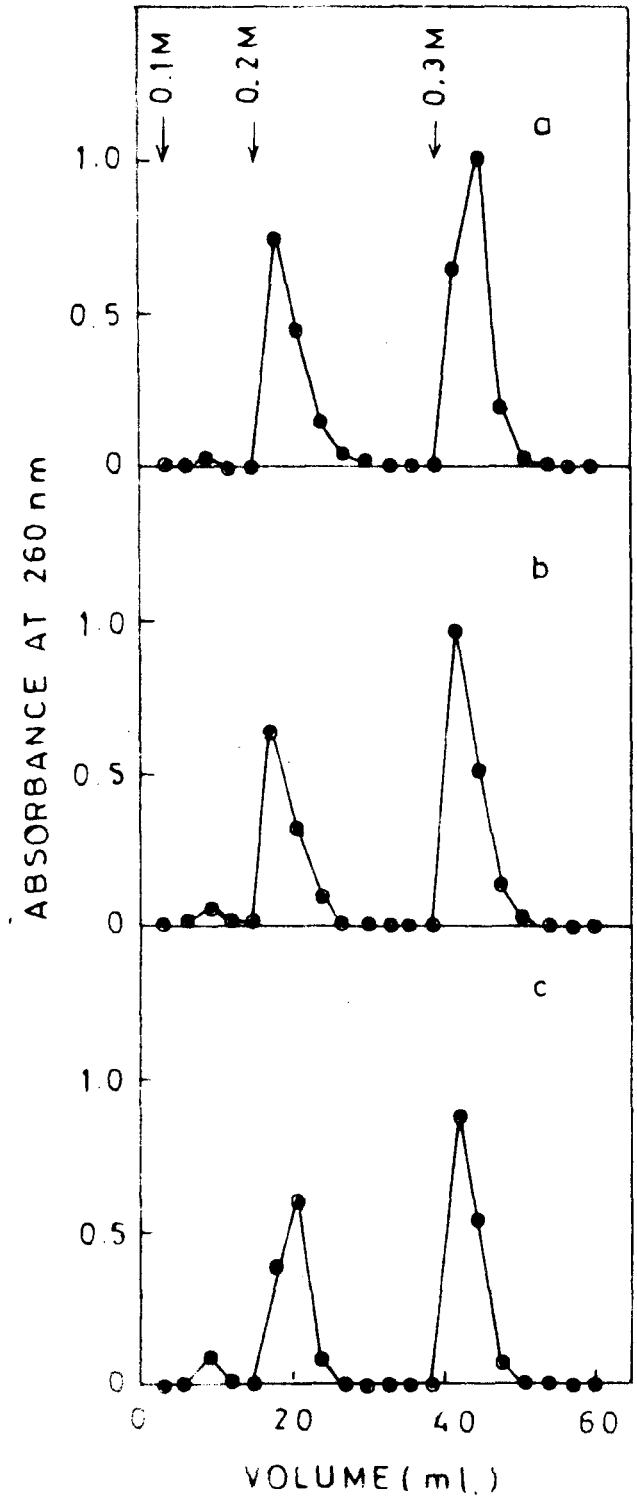
TABLE - 9

ALKALINE HYDROLYSIS OF DIMETHYL SULFATE TREATED DNA

DNA/DMS MOLAR RATIO	TIME OF ALKYLATION (Hours)	NaCl Conc.	% DNA DENATURED (CALCULATED FROM FIGURE)	ALKALINE HYDROLYSIS OF ALKYLATED DNA (u MOLES ACID SOLUBLE DNA NUCLEOTIDE)	ALKALINE HYDROLYSIS OF DEPURINATED DNA (u MOLES ACID SOLUBLE DNA NUCLEOTIDE)
No DMS	-	0.01M	0.0	0.005	0.005
1:1	1	0.1M	9.8	0.005	0.033
1:4	1	0.1M	22.5	0.005	0.516
1:8	1	0.1M	46.0	0.110	0.580
1:4	2	0.01M	50.0	0.013	-
1:4	5	0.01M	54.0	0.019	-
1:4	10	0.01M	82.0	0.022	-
1:4	10	0.1M	51.0	0.010	-

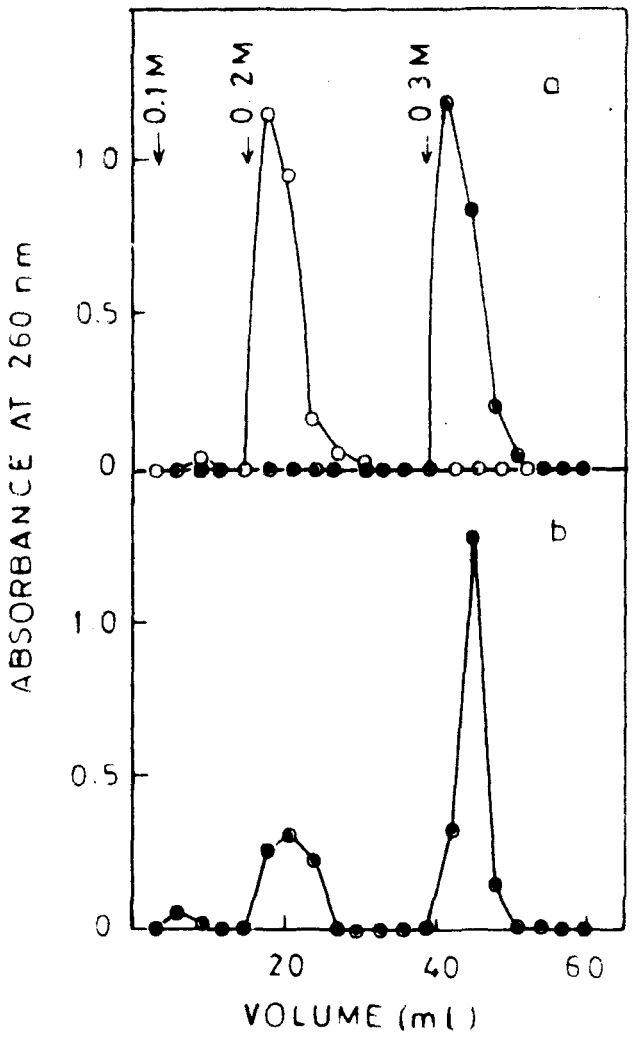
Per cent denatured DNA was calculated from the data of the Figure. Hyperchromicity of DNA on denaturation was taken into account before calculating the percentage. See text for details of alkaline hydrolysis of alkylated and depurinated DNA.

Figure - 26. <u>Chromatography on hydroxyapatite of alkylated DNA incubated for various periods after removal of the alkylating agent</u> - DNA was alkylated with DMS in a DNA nucleotide/DMS molar ratio of 1:4. The alkylated DNA was dialysed against 50 volumes of TNE (0.01M Tris-HCl, 0.1M NaCl, 2×10^{-4}M EDTA; pH 7.5) at 4° before incubating at room temperature for various periods. The pattern is shown for dialysed alkylated DNA incubated for (a) 0.0 hr., (b) 24 hr. and (c) 48 hr.


0.1M
0.2M
0.3M
a
b
c
1.0
0.5
0
ABSORBANCE AT 260 nm
0
20
40
60
VOLUME (ml.)

In order to determine the effect of ionic strength on denaturation the experiment shown in Fig. 25 was done. DNA was alkylated for different periods in 0.01M NaCl and subjected to hydroxyapatite chromatography. A DNA nucleotide/DMS molar ratio of 1:4 was chosen since minimum hydrolysis of this DNA is caused with alkali. Almost all DNA molecules are denatured after incubation for 10 hours in 0.01M NaCl (Fig. 24e) while only 50% molecules are denatured in 0.1M NaCl in the same period (Fig. 24f). A control DNA sample kept in presence of 0.01M NaCl at room temperature for 10 hours did not show any production of denatured molecules. In all experiments where alkylation was done in presence of 0.01M NaCl or during the hydroxyapatite chromatography of depurinated DNA some A_{260} absorbing material was eluted with 0.1M phosphate buffer which corresponded to mononucleotides. In Fig. 26 is shown the effect of incubating the alkylated DNAs at room temperature after removal of the unreacted dimethyl sulfate by dialysis. Such DNAs did not show any further denaturation when kept at room temperature upto 49 hours (Fig. 26c,d). This implies that the extent of denaturation of DNA as a result of alkylation with DMS was dependent on the continued presence of the alkylating agent. Once the alkylating agent was removed no more strand separation occurred.

Figure - 27. Chromatography of cross linked DNA on hydroxyapatite - DNA was cross linked by HNO_2 as described in the text. Cross linked DNA was heated at 100° for 7 minutes and cooled rapidly in ice. 600 ug of this DNA was chromatographed on a hydroxyapatite column as described in Fig. 22. The elution pattern is shown for (a) native and denatured DNA and (b) cross linked heat denatured DNA.


0.1M
0.2M
0.3M
a
b
1.0
0.5
0
1.0
0.5
0
ABSORBANCE AT 260 nm
20
40
60
VOLUME (ml)

Cross linking of DNA

Cross linked DNA prepared by treatment of calf thymus DNA with nitrous acid was chromatographed on hydroxyapatite column. As expected cross linking did not cause any denaturation of DNA and made it resistant to denaturation by heat (Fig. 27). Only 25% molecules of cross linked DNA were denatured contrary to 100% denaturation of native DNA by the same treatment. When cross linked DNA was depurinated by heating at pH 3.5 at 70° and chromatographed on hydroxyapatite it did not bind to the column and was eluted with the flow through eluate. Possibly cross linked DNA on depurination loses its rigidity which is the basis of binding to hydroxyapatite.

Enzymatic degradation of DNAs alkylated at different DNA nucleotide/DMS molar ratios

Fig. 28 shows the hydrolysis of alkylated and depurinated DNAs alkylated with DMS in different molar ratios. The degree of hydrolysis by the enzyme can be related to the degree of denaturation. The maximum acid soluble material produced by enzyme increased with increase in DMS ratio used for alkylation. The alkylation of DNA in DMS molar ratio 1:4, 1:2 and 1:1 cause denaturation of 22.5, 15 and 10 percent molecules of alkylated DNA respectively. Corresponding enzymatic hydrolysis cause production of 20, 14 and 8 percent acid soluble material from these

Figure - 28. Enzymatic degradation of alkylated DNA and depurinated DNA obtained from alkylated samples - Depurinated DNA was prepared by mild heating of alkylated DNA as described in the text. Enzyme was assayed using increasing concentrations of Fraction IV (sp. act. 58). (a) 1:4 DNA nucleotide/DMS molar ratio, (b) 1:2 DNA nucleotide/DMS molar ratio and (c) 1:1 DNA nucleotide/DMS molar ratio. Alkylated DNA (O—O), depurinated DNA (●—●).

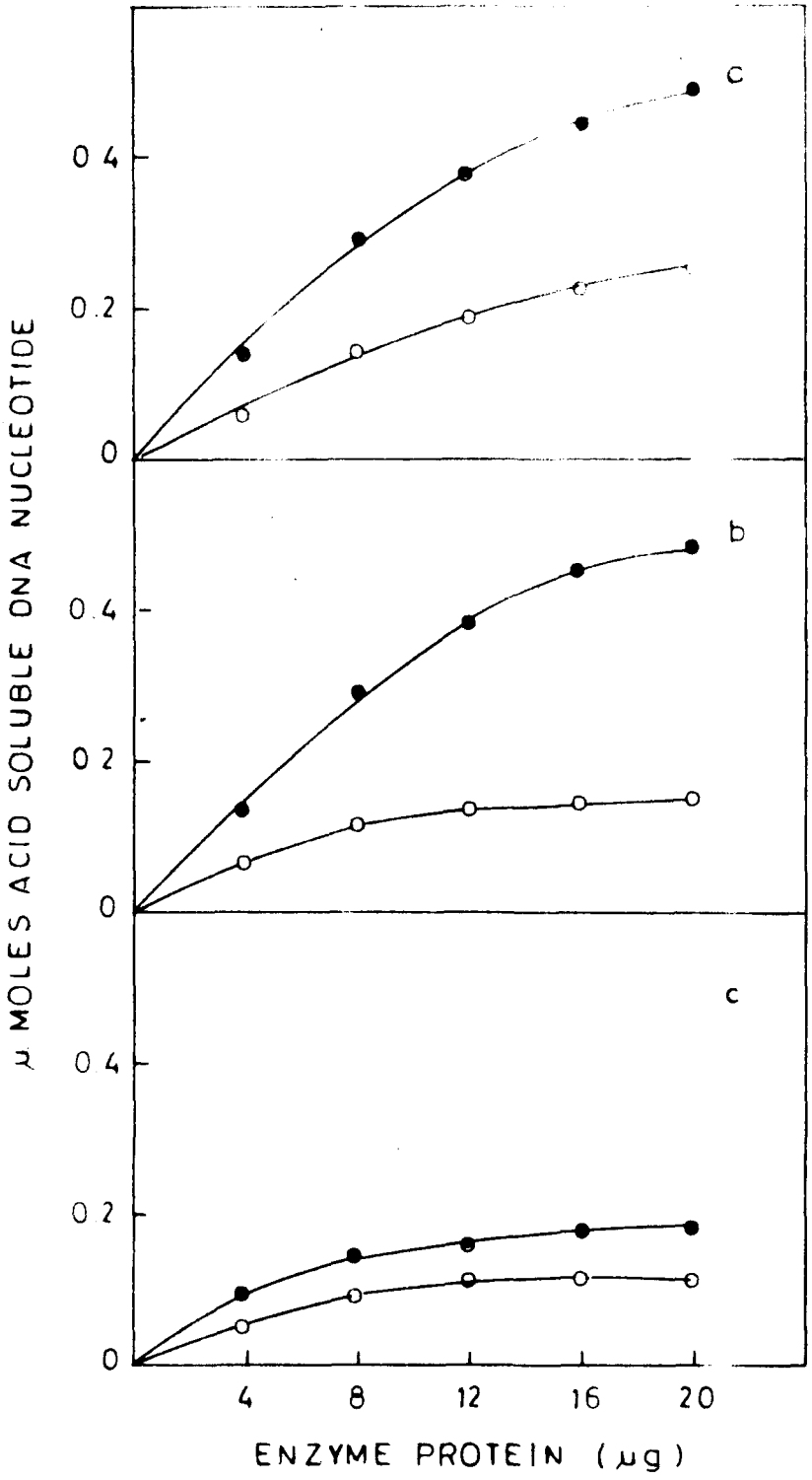
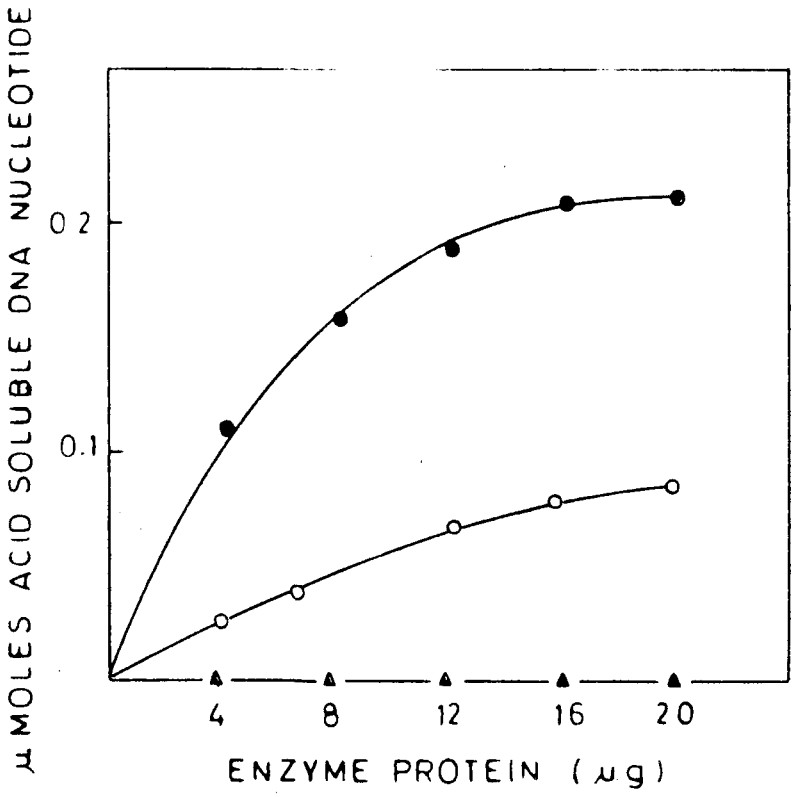

μ MOLES ACID SOLUBLE DNA NUCLEOTIDE
0.4
0.2
0
a
0.4
0.2
0
b
0.4
0.2
0
c
4
8
12
16
20
ENZYME PROTEIN (μg)

Figure - 29. Enzymatic degradation of cross linked DNA - Reaction mixture in 1 ml contained 200 ug DNA, 1 mM $MgCl_2$, 1mM mercaptoethanol and Fraction IV at indicated concentrations. Cross linked DNA (▲—▲); cross linked heat denatured DNA (○—○); cross linked depurinated DNA (●—●).


μ MOLES ACID SOLUBLE DNA NUCLEOTIDE
0.2
0.1
4
8
12
16
20
ENZYME PROTEIN (μg)

DNA preparations respectively. The values correspond well with the percentage of molecules denatured. The percent denaturation of depurinated DNAs obtained from DNAs alkylated with DNA nucleotide/DMS molar ratios of 1:4, 1:2 and 1:1 was 95, 80 and 10 respectively. Acid soluble material liberated on enzymatic hydrolysis of these DNAs was 45, 45 and 10 percent respectively. These results are compatible with our assertion that the pea seed nuclease is specific for denatured DNA and the activity upon native DNA is due to the presence of partially denatured regions in the normal DNA. The extent of degradation increases as the degree of denaturation increases. When the partial single strand character of native DNA is suppressed the activity on native DNA also decreases. When DNA is cross linked with nitrous acid the single stranded regions presumably do not exist any more for it is not at all degraded by enzyme (Fig. 29). Since cross linking made DNA resistant to denaturation by heat and only 25% molecules showed strand separation the hydrolysis of cross linked DNA heated at 100° was low. Cross linked DNA when depurinated by heating at pH 3.5 and treated with enzyme showed significant degradation. This is presumably due to the fact that depurination removes the cross links since cross linking takes place through the bases.

TABLE - 10

DEGRADATION OF DIFFERENT DNA SUBSTRATES WITH DNase I AND PEA SEED NUCLEASE

The different DNA substrates were prepared as described in "methods". 0.3 unit/ml of fraction IV (sp. act. 62) and 2 ug/ml of deoxyribonuclease were used. With pancreatic DNase I the extent of degradation of native DNA is taken as 100% and with pea seed nuclease the degradation of denatured DNA is taken as 100%.

SUBSTRATE	PERCENT OF NATIVE DNA DEGRADING ACTIVITY	PERCENT OF DENATURED DNA DEGRADING ACTIVITY
	PANCREATIC DNase I	PEA SEED NUCLEASE
NATIVE DNA	100	7
DENATURED DNA	101	100
ALKYLATED DNA	94	99
DEPURINATED DNA	94	116
CROSS LINKED DNA	82	0.00

Degradation of different DNA substrates with DNase I and pea seed nuclease

Table 10 summarizes the degradation of modified DNAs with pancreatic DNase I and pea seed nuclease. Pancreatic DNase was assayed like pea seed nuclease except that incubation mixture contained 10mM Mg^{++}. All the DNA substrates were degraded at an equal rate by DNase I. The degradation of native DNA and cross linked DNA by pea seed nuclease was quite low in comparison with that of denatured DNA or alkylated and depurinated DNA. The results are consistant with the conclusion that the pea seed nuclease is specific for single stranded DNA and the alkylated and depurinated DNA was degraded because certain amount of denaturation may occur as a result of alkylation.

CHAPTER - IV

D I S C U S S I O N

DISCUSSION

Studies with crude extract

Cellular DNA is susceptible to several kinds of damage, such as irradiation, interaction with chemical mutagens and carcinogens and depurination. DNA repair systems operate in cells maintaining high molecular weight and biological activity of DNA. Excision repair model[66] of U.V.-irradiated thymine dimer containing DNA involves an endonucleolytic event as the first step. Similar mechanism may be operative for the repair of alkylated and depurinated DNA[15]. Endonucleases specific for alkylated and apurinic sites in DNA have been shown to exist in bacteria[11,14], mammalian cells[18,19] and plants[15,16]. However, no plant nuclease specific for apurinic or alkylated sites have been characterized to date. An effort was therefore made to identify nucleases in plant material that might prefer alkylated, depurinated and single stranded DNAs. Experiments carried out with crude extract from germinating pea seeds (Fig. 1,2 and 3) identified a nuclease activity in these extracts which preferred alkylated, depurinated and denatured DNA over native DNA. Depurinated DNA can spontaneously arise from alkylated DNA[15] by depurination of alkylated bases. In addition extensive depurination may lead to denatured regions in the double stranded molecule. It was therefore difficult to

determine whether the preferential degradation of the modified and denatured DNA was due to a single enzyme or due to a multiple of activities. The enzyme(s) was therefore purified and characterised to determine its specific substrate. It should be further noted (Fig.3) that maximum degradation of depurinated DNA was higher compared to that of denatured DNA. The method used to prepare depurinated DNA here leads to denaturation[14]. The crude extract may therefore be containing an additional nuclease that specifically attacks DNA at apurinic sites.

Purification and characterisation of pea seed nuclease

In view of ambiguity of results obtained with crude extracts, the enzyme(s) responsible for degradation of alkylated, depurinated and denatured DNA was purified. Since cotyledons contain a considerable amount of fat which interferes with the purification procedure, the purification was attempted using extracts of germinated embryo axis only. On ammonium sulfate fractionation most of the nuclease active on denatured DNA is precipitated between 40-80 percent saturation. DEAE-cellulose chromatography of this fraction yielded a discrete peak of nuclease active on single stranded DNA. Most purification was achieved on the Phosphocellulose column where much of the unwanted protein was removed. This fraction (Fraction IV) was concentrated and used for characterisation of the enzyme. On polyacrylamide gel electrophoresis it

showed three distinct bands (Fig. 6). Attempts to identify the enzymatically active band were unsuccessful since the enzyme lost activity on electrophoresis.

That the purified enzyme degrades single stranded DNA was evidenced by several experiments described in the results. Both heat and alkali denatured DNAs are hydrolysed at a several fold greater rate than the native molecule (Fig. 7-10, Table 10). Similarly depurinated DNA which was single stranded under our conditions was degraded at a much higher rate. Further evidence is obtained from the experiments on the degradation of alkylated DNA by the enzyme. Increasing denaturation of the DNA as a result of higher ratios of alkylating agent resulted in increased enzymatic hydrolysis of the alkylated DNA (Fig. 28). Under the usual assay conditions the maximum hydrolysis of native DNA was only 7-10 percent (Fig. 9, Table 10) whereas 100 percent of the input denatured DNA could be digested. That these two enzymatic activities were due to a common protein was suggested by several observations. Both activities were present in all stages of purification. Neither activity was inhibited by EDTA while both show inhibition by 8-hydroxyquinoline. Both were stimulated by Ca^{++} and Mg^{++} (Table 5). 2-mercaptoethanol was inhibitory to both to the similar extent (Fig.16). It is realised, however, that further purification and identification of the active band on

polyacrylamide gel electrophoresis are required to establish firmly that a single enzyme is responsible for both the activities.

In constrast with the action of the crude extract the purified enzyme was equally active on depurinated and denatured DNA (Fig. 8). These results suggest that the crude extract may contain an additional nuclease activity that may be specific or preferentially active on depurinated DNA. This activity is presumably removed during purification. The purified enzyme which is specific for single stranded DNA thus cannot differentiate between single stranded and single stranded depurinated DNA.

Indirect evidence has been presented to support the contention that the activity of the pea seed nuclease on native DNA is due to the presence of denatured regions in the native molecule which may be present naturally or may have been introduced during the isolation of DNA. As shown in Fig. 9 only 7 percent of native DNA was hydrolysed and when an equal amount of substrate was further added, another 7 percent hydrolysis takes place after which the reaction did not proceed. The low level of activity on native DNA is not due to the contamination of the substrate with denatured DNA. This was confirmed by hydroxyapatite chromatography of native DNA where no single stranded material was obtained. The work of Von-Hippel and coworkers[116,117,124] has earlier demonstrated that A, T-rich regions of double stranded DNA possess a degree of single stranded character. By manipulating reaction conditions such as

temperature and ionic strength it is possible to enhance or suppress the single stranded character of such regions. As shown in Fig. 12 the optimum temperature of the enzyme for the degradation of denatured DNA is 45° whereas it is 60° with native DNA. In addition when reaction was carried out at elevated temperature above 37° increasing degradation of double stranded DNA was observed in both rate and extent. Since the structural breathing is augmented at temperatures above 37° but below the Tm, due to greater strand separation in A,T-rich areas, the results suggest that the degradation of double stranded DNA by the enzyme is mainly directed towards the limited areas of partial denaturation. The experiment on the effect of salt on enzymatic activity (Table 4) also tends to support this observation. Salt stabilises the secondary structure of DNA, by suppressing the structural breathing[116,117]. It was observed that the enzymatic degradation of native DNA was suppressed to a much greater extent in the presence of salt than denatured DNA.

The pea seed nuclease which is similar to the nucleases specific for single stranded DNA from N. crassa[43], A.oryzae[45] and U. maydis[69] did not show any absolute requirement for a metal ion. It, however, differs in its absence of inhibition by EDTA while all fungal nucleases are strongly inhibited by EDTA, but are reactivated on subsequent addition of divalent cations particularly Co^{++}, Zn^{++} and Mg^{++}. The pea seed nuclease on the

other hand shows activation by Ca^{++} and Mg^{++} which can be reversed on the addition of EDTA. The enzyme also did not seem to require an -SH group for its activity since it was not inhibited by sulfhydral reagents. The metal ions interact with the enzyme rather than with the DNA (Fig.15); presumably the metal ion confers on the enzyme a suitable conformation for the interaction with the substrate. The inhibition by 2-mercaptoethanol of the degradation of both native as well as denatured DNA could be due to either reduction of disulfide bridges in the enzyme or interaction with the enzyme bound metal[44]. The molecular weight of the pea seed nuclease as determined by molecular sieve chromatography on sephadex G-200 was 42,000 daltons. It is, however, realized that to obtain an accurate figure other methods such as electrophoresis, and sedimentation velocity have to be used. Incidently the single stranded DNA specific nuclease from Ustilago maydis also has a molecular weight of 42,000[69]. However, the pea seed nuclease differs from it in other respects.

The optimum pH of pea seed nuclease is 7.5 in tris-HCl buffer and it is active over a broad range of temperature from 30 to 65°. In contrast the single stranded DNA specific nucleases, S_1 from A. oryzae and from mung bean are optimally active in the acidic range. The importance of these enzymes as a tool for the probe of low melting regions of DNA has already been recognized. The inclusion of the pea seed nuclease in this category of enzymes can

provide us with a class of enzymes active under more versatile range of conditions.

Although no specific physiological role for single stranded DNA specific nucleases has been assigned several cellular processes can be considered where these may be involved. These are DNA recombination[125-127] replication and repair. The pea seed enzyme is capable of degrading native DNA at partially single stranded regions thereby introducing single stranded breaks which are necessary for the exchange event. The production of free DNA ends and subsequent enzymatic attack is an essential part of the recombination process where the pea seed nuclease may be implicated. With respect to DNA replication the enzyme can perhaps recognize unpaired regions or mismatched base pairs in replicating DNA and may have therefore a vital proof reading function. The nuclease may also be able to attack tertiary twists in the DNA ahead of the replicating fork, thereby permitting a facilitated access of replication proteins to the template as well as the unwinding of the DNA duplex. At present we have no idea as to the length of the denatured region in the duplex before it can be recognised by the pea seed nuclease. However, it is possible that the enzyme may be able to recognize and cleave DNA at the site of an unpaired base produced by depurination of its complementary purine. Depurination also happens to be a secondary lesion of several kinds of DNA damage such as alkylation and U.V-irradiation[20] . It could thus have a

role in DNA repair as well.

Studies on the effect of alkylation upon secondary structure of DNA

Alkylation transformed the DNA into a suitable substrate which was degraded much more efficiently than the native molecule. It was of interest therefore, to determine the change brought about in the secondary structure of DNA on alkylation. Hydroxyapatite chromatography was employed for the purpose. The results demonstrated that alkylation may lead to denaturation of the duplex under conditions where little or no depurination occurs. The extent of single stranding depends on the concentration of the alkylating agent and the period of alkylation. In the presence of higher ionic strength the extent of denaturation is decreased. N-7 position of guanine and N-3 position of adenine are the major sites of alkylation by dimethyl sulphate (DMS), the alkylation agent used in our studies[128] and it leads to the production of quarternized alkylated bases carrying a net positive charge. In their studies on the effect of alkylation on the binding of ethidium to duplex DNA Hsiung et al.[129] observed a relationship between the decrease in flouroescence of intercalated ethidium and the extent of alkylation of DNA at pH 7.0. This was attributed to a charge repulsion between an alkylated quarternized base and positively charged ethidium which resulted in a decrease in the number of ethidium binding sites. These authors also suggested that partial

denaturation at higher pH values may occur through the disruption of hydrogen bonding due to a base catalysed imidazole ring opening. It is suggested that the positive charges of the quarternised alkylated bases alone through repulsion may adversely effect the forces stabilizing the secondary structure of DNA. This possibility is consistant with the preventive effect of salt on denaturation of alkylated DNA. Regardless of the precise interpretation of our results it is clear that the alkylation of DNA with DMS makes the secondary structure of DNA relatively unstable.

The enzymatic experiments carried out with modified substrates such as alkylated, depurinated or cross linked DNA were designed to show that the activity of the enzyme is effected by the extent of denaturation of the modified substrates (Fig. 28,29). It was observed that the rate and extent of hydrolysis of these substrates by the pea seed nuclease correlate with the degree of denaturation and reflect the requirement of the enzyme for polynucleotides without an ordered secondary structure. Cross linking of DNA by nitrous acid presumably completely suppresses the existing denatured areas in the duplex DNA. This was evidenced by the fact that cross linked DNA was completely refractory to the action of the pea seed nuclease (Fig. 29). Thus by several considerations outlined above it can be concluded that the pea seed nuclease is specific for single stranded DNA or regions in duplex DNA that bear single stranded character.

The importance of endonucleases specific for single stranded DNAs has been widely recognized in many laboratories. The pea seed nuclease can be a complement to the already existing enzymes of this nature as has been discussed earlier. Some of the uses where the pea seed enzyme can be utilized with benefit are to (1) quantitate the nucleic acid hybridization, (2) analyse heteroduplex DNAs, (3) isolate rapidly annealing regions in single stranded DNA, (4) detection of gaps in duplex DNA, (5) analysis of superhelical DNA and (6) possibly the analysis of mismatched or unpaired bases in duplex DNA.

CHAPTER - V

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

1. A nuclease activity was identified in the crude extracts of germinating pea seeds that degraded denatured, alkylated and depurinated DNA at a significantly higher rate than native DNA. The activity was found to be present in dry and imbibed seeds. There was no significant change in its specific activity over a germination period of 120 hours.

2. The enzyme was purified about 150-fold on the basis of its ability to degrade single stranded DNA, using ammonium sulfate fractionation, DEAE-cellulose and Phosphocellulose chromatography. The final Phosphocellulose fraction (Fraction IV) was not homogeneous as three discrete protein bands were observed on disc gel electrophoresis.

3. From exclusion chromatography on sephadex G-200 the molecular weight and Stoke's radius of pea seed nuclease was calculated to be 42,000 and 8.19×10^{-7} cm.

4. The partially purified enzyme degraded single stranded DNA at a 15 to 20 fold higher rate than native DNA. The hydrolysis of single stranded and depurinated DNA by partially purified enzyme was found to be at comparative

levels. That the activity of the purified preparation on double and single stranded DNA is due to a single enzyme is suggested by several criteria.

5. The Km value for the enzyme as determined in terms of acid soluble DNA nucleotides formed by the hydrolysis of single stranded DNA was of the order 3.33×10^{-6}M.

6. The enzyme shows activity over a broad range of pH but was most active between pH 6.5 to 8.0. At pH 7.5 the enzyme was maximally active in tris-HCl buffer, while in other buffers of same molarity and pH the enzyme shows decreased activity.

7. A broad range of temperature tolerance was shown by the enzyme. The maximum hydrolysis of denatured DNA was observed at 45° while with native DNA the temperature optima was 60°. The enzyme is fairly stable and retains unchanged activity when stored frozen in the presence of 10% glycerol in 0.02M tris-HCl buffer, pH 7.5. Slow decrease in enzyme activity was observed as a function of time when enzyme was incubated at 37° and 45°. However at 50°, half of the activity was lost within two hours.

8. The small extent of hydrolysis of native DNA is suggested to be due to the degradation of partially denatured areas in the native molecule and not due to a contamination by single stranded molecules in the native DNA preparation. This was concluded on the basis of experiments in which

native DNA when treated with nuclease at elevated temperatures, showed increased hydrolysis both in extent and rate. This indicated that the enzymatic activity was directed towards low melting or A, T-rich regions in native DNA. Higher ionic strength suppresses the structural breathing in native DNA. The enzymatic activity of the nuclease on native DNA was found to be reduced by approximately 90% in the presence of 0.2M NaCl. This property further supported the idea that the degradation of native DNA by the enzyme was mainly directed towards partially denatured areas.

9. The nuclease does not show an absolute requirement for added divalent cations. However the addition of Mg^{++} and Ca^{++} results in 40% and 60% stimulation respectively. Among the chelating agents 8-hydroxyquinoline was inhibitory whereas EDTA had no effect upon the enzymatic activity. The observed inhibition of the pea seed nuclease in presence of 8-hydroxyquinoline but not with EDTA as well as the lack of an absolute requirement for the added divalent cation suggest that free metal is not required for enzyme function and the inhibition by 8-hydroxyquinoline is probably through direct interaction with the enzyme possibily with a bound metal. Combined addition of Ca^{++} and Mg^{++} to the reaction mixture did not show synergistic effect. EDTA causes the

reversal of stimulation by Ca^{++} and Mg^{++}. The enzyme does not seem to require an -SH group for its activity. Addition of yeast RNA and several monoribonucleotides did not show inhibition of enzyme activity.

10. The study of products of enzymatic hydrolysis of denatured DNA on sephadex G-100 show that oligonucleotides are produced during the course of reaction and are progressively reduced to smaller fragments untill virtually the entire population of single stranded DNA is rendered acid soluble. The chromatographic profile obtained with the digests of enzymatic hydrolysis was consistant with an endonucleolytic mode of action.

11. The preferential degradation of alkylated DNA by both crude as well as purified enzyme was further investigated by studying the effect of alkylating agents on the secondary structure of DNA. Native DNA on its treatment with dimethyl sulfate undergoes strand separation as determined by hydroxyapatite chromatography. The extent of denaturation depends upon the DNA nucleotide/DMS molar ratio. However, the presence of a higher salt concentration has a preventive effect on alkylation induced denaturation. The hydrolysis of alkylated DNA by the enzyme is due to the denaturing effect of the alkylating agent.

12. Cross linked DNA is resistant to the action of pea seed

nuclease. Since cross linking may presumably suppress the single stranded character of native DNA this fact further strengthens the indication that nuclease recognizes the partially denatured areas in the native molecule.

The importance of single strand specific deoxyribonucleases has been recognised in many laboratories. The higher selectivety of pea seed nuclease towards single stranded DNA (whether modified or otherwise) and denatured or low melting regions in native DNA, stability at a wide range of temperatures and ionic strength, a neutral pH optima and ease of preparation of this nuclease endow this enzyme with optimal qualities required for a nuclease to be used as an analytical tool. With respect to its physiological role the pea seed nuclease may be implicated in several processes of biological importance such as DNA repair, replication and recombination. As a biochemical tool it complements the other single strand DNA specific nucleases such as the mung bean nuclease and S_1 nuclease of Aspergillus oryzae that have already been reported to be of wide utility.

CHAPTER - VI

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CHAPTER - VII

PUBLICATIONS AND PRESENTATIONS

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1. Altaf A. Wani and S.M. Hadi "A nuclease specific for depurinated DNA in germinating pea seeds". Ind. J. Biochem. Biophys., Vol. 13, No. 3, pp 307-309 (1976).

2. Altaf A. Wani and S.M. Hadi "Effect of dimethyl sulfate upon the secondary structure of DNA". Experientia (Communicated).

3. S.M. Hadi, N.S. Ahmad and Altaf A. Wani "An alkaline nuclease specific for single stranded DNA in army worm (Spodoptera litura)". Insect Biochemistry (Communicated).

4. Altaf A. Wani and S.M. Hadi "A nuclease specific for depurinated DNA in germinating pea seeds". Presented at 44th Annual General Meeting of Society of Biological Chemists (India) held at Calcutta. Ind. J. Biochem. Biophys., Vol.13, p. 18 (1975) Suppl.

5. Altaf A. Wani and S.M. Hadi "Effect of dimethyl sulfate upon the secondary structure of DNA". Presented at 46th Annual General Meeting of Society of Biological Chemists (India) held at Madras (1977).

6. S.M. Hadi and Altaf A. Wani "Nuclease for DNA apurinic sites in germinating pea seeds". Abstracts, 10th International Congress of Biochemistry, Hamburg, Germany (1976) p. 629.

7. S.M. Hadi and Altaf A. Wani "Nuclease specific for single stranded DNA in germinating pea seeds". Abstracts, 1st Congress of Federation of Asian and Oceanian Biochemists, Nagoya, Japan (1977).

8. S.M. Hadi and Altaf A. Wani "Nuclease specific for single stranded DNA in germinating pea seeds". International Symposium on Biomolecular structure, conformation, function and evolution. (Jan. 4-7, 1978) University of Madras, India. Accepted for presentation.